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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: COILED-COIL HETERODIMER METHODS AND COMPOSITIONS FOR THE DETECTION AND PURIFICATION OF EXPRESSED PROTEINS

(57) Abstract

Methods and compositions employing complementary heterodimer-subunit peptides capable of forming a α-helical coiled-coil heterodimer for affinity purification and detection of fusion proteins.

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COILED-COIL HETERODIMER METHODS AND COMPOSITIONS
FOR THE DETECTION AND PURIFICATION OF EXPRESSED PROTEINS

5   This application claims priority to U.S. Patent Application Serial No. 08/540.397
filed 6 October 1995. herein incorporated by reference.

Field of the Invention

          This invention relates to the use of a pair of peptides capable of forming α-helical
10   coiled-coil heterodimers for the purification and detection of fusion proteins containing one
of the peptides of the pair.

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Background of the Invention

One of the major commercial applications of biotechnology is recombinant
expression of proteins. Several recombinant proteins, including epoetin α, G-CSF,

25   somatotropic, insulin, tPA, urokinase and prourokinase, various interferons, EPO, and
specialty enzymes have well established commercial markets (Bioprocess Engineering).

There are three general technical hurdles in bringing a protein to recombinant
production. The protein's coding sequence must be obtained and the gene cloned in a
suitable expression vector; a host cell and culture conditions favorable for expressing and

30   preferably secreting the protein in active form must be found; and the expressed protein
must be isolated and, in some case, enzymatically processed, to produce a desired final
product, *e.g.*, one suitable for pharmaceutical use.

For purposes of finding conditions that favor high levels of protein expression and
secretion, it is desirable to be able to monitor expression, levels of expression, and

localization of expressed proteins for a given expression system. Ideally. such monitoring
can be done without modifying the expressed protein. and can be performed in an *in situ*
format. as well as various *in vitro* formats, such as dot blot or Western blot analysis.

For purposes of isolating expressed proteins. it is desirable to generate the protein
5   in a form that lends itself to one-step affinity purification. An approach that has been
proposed previously for this purpose is to express the protein in tandem with a small
peptide antigen segment. One then purifies the expressed protein on the basis of its specific
binding to a support matrix carrying an antibody specific against the antigen. For large-
scale production. this approach requires amounts of antibody commensurate with the
10   amount of protein produced. which may add significantly to the cost of protein production.
The need for antigen competition to release the bound protein from the solid support may
also add to the cost of the purification step.

Summary of the Invention

15   The invention includes an expression vector composed of a replication segment
which permits replication of the vector in a selected host cell. and an expression cassette.
The cassette contains, in a 5'-3' direction. a promoter functional in the host cell and a
coding segment. The coding segment. in turn. contains a heterologous DNA coding site
and a DNA region encoding a first heterodimer-subunit peptide capable of forming an α-
20   helical coiled-coil heterodimer with a complementary second heterodimer-subunit peptide.
Further. the coding segment may contain. disposed between the coding site and the DNA
region. a cleavage sequence. in frame with the DNA region and encoding an amino acid
sequence that provides a target for chemical or enzymatic cleavage. The coding segment
may be oriented with either its coding site or DNA region adjacent the promoter.

25   In a preferred embodiment. one of the (first and second heterodimer-subunit
peptides) contains at least two heptad amino acid repeat sequences having the form gabcdef.
where positions a and d of each amino acid repeat sequence are selected from the group
consisting of leucine. isoleucine and valine. and positions e and g of each amino acid repeat
sequence are selected from the group consisting of aspartic acid and glutamic acid. The
30   other of the (first and second heterodimer-subunit peptides) contains at least two heptad
amino acid repeat sequences having the form g'a'b'c'd'e'f'. where positions a' and d' of
each amino acid repeat sequence are selected from the group consisting of leucine.
isoleucine and valine. and positions e and g of each amino acid repeat sequence are selected
from the group consisting of lysine. arginine and histidine. In this preferred embodiment.

each of the corresponding d/a' and a/d' pairs in complementary heptads consists of residues where one of the residues is a valine and the other is selected from the group consisting of leucine and isoleucine.

The cloning site preferably includes a multiple cloning site (MCS) at which a heterologous DNA coding region can be inserted. Such a heterologous DNA coding region typically encodes a selected polypeptide of interest. The DNA encoding the selected polypeptide is typically inserted at the cloning site such that it is expressed in frame with coding sequences in the DNA region encoding the first heterodimer-subunit peptide are the sequences represented by SEQ ID NO:2 and SEQ ID NO:4.

In a related aspect, the invention includes a coding segment, for use in an expression vector suitable for expressing heterologous proteins in a host cell (such as the vector described above). The segment includes a heterologous DNA coding site and a DNA region encoding a heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a complementary heterodimer-subunit peptide, as described above.

Further, the segment may contain, between the coding site and the DNA region, a cleavage sequence, in frame with the DNA region, encoding an amino acid sequence comprising a target for chemical or enzymatic cleavage. The coding site may be situated either 5' or 3' of the DNA region. The cloning site and coding sequences include the embodiments described above in relation to the expression vector.

Also included in the invention is a fusion protein encoded by the coding segment described above or produced by an expression vector containing such a coding segment, such as the expression vector described above. The fusion protein is composed of a polypeptide having a functional activity (i.e., a selected polypeptide of interest), and a heterodimer-subunit peptide (i.e., a coil peptide, such as the E-coil, 0993, K-coil or 0994 peptide described herein) of the type described above, attached to the N- or C-terminus of the polypeptide.

Also included in the invention is a reagent for detecting the presence of the expressed fusion protein. The reagent is composed of a second heterodimer-subunit peptide (second coil peptide) capable of forming an α-helical coiled-coil heterodimer with the heterodimer-subunit peptide (first coil peptide) in the fusion protein. The reagent is useful for detecting expression of a fusion protein containing a heterodimer-subunit peptide, such as described above, by forming a heterodimer or heterodimer complex between the heterodimer-subunit peptide contained in the fusion protein and the detection reagent, and detecting the presence of heterodimer formation.

A kit for detecting the expression of a selected polypeptide includes an expression vector containing the above-described coding segment, for expressing a fusion protein containing the heterodimer-subunit peptide, and the above detection reagent.

In still another aspect, the invention includes an affinity matrix composition for the
5   purification of a selected polypeptide. The composition includes a solid support, and attached to said support, a heterodimer-subunit peptide (coil peptide, e.g., SEQ ID NO:4 or SEQ ID NO:27) of the type described above. The composition is useful in a method for purifying a selected expressed polypeptide, as described below.

The invention also includes a method of purifying a selected expressed polypeptide.
10 The method includes the steps of (i) expressing, in a suitable host-cell expression system (e.g., an E. coli or Yeast expression system), a fusion protein containing the selected polypeptide in tandem with a first heterodimer subunit (e.g., SEQ ID NO:2 or SEQ ID NO:26), (ii) obtaining, from the host cell expression system, a suspension containing said fusion protein, and (iii) passing this suspension over an affinity matrix composition as
15 described above. The affinity matrix is then washed while the fusion protein remains bound to the affinity matrix via the α-helical coiled-coil heterodimer. The washing includes, in either order, (a) a salt wash step comprising washing the affinity matrix with a solution having a pH of between about 5.5 and about 8.0 and containing between about 0.1 M and about 1 M salt (e.g., sodium chloride, potassium chloride, sodium acetate, ammonium
20 chloride, ammonium acetate, sodium perchlorate, potassium perchlorate, sodium phosphate, and potassium phosphate), and (b) an organic wash step comprising washing the affinity matrix with a solution having a pH of between about 5.5 and 8.0 and containing between about 50% and about 100% of an organic solvent (e.g., methanol, ethanol, isopropanol, tetrahydrofuran, trifluoroethanol, and acetonitrile). After washing, the selected polypeptide
25 is released from the matrix either by elution or by enzymatic cleavage. Preferably, the elution solution has a pH of about 3.0 or lower, or about 10.0 or higher. Exemplary elution solutions contain between about 20% and about 80% of an organic solvent.

A kit for obtaining an expressed protein in purified form includes the above expression system, for expressing a fusion protein having a heterodimer-subunit peptide of
30 the type described above, and an affinity matrix composition of the type described above, for affinity binding of the expressed fusion protein to the solid support in the composition.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is read in conjunction with the accompanying drawings.

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Brief Description f the Figures

Figures 1A. 1B. 1C. 1D. 1E and 1F show a scheme for affinity purification according to the method of the invention.

Figure 2 shows helical wheel representations of peptides 0993 (SEQ ID NO:26) and
5   0994 (SEQ ID NO:27) in an α-helical heterodimer configuration.

Figures 3A. 3B, 3C. 3D and 3E show a schematic representations of adjacent
heptads of two heterodimer-subunit peptides in a parallel configuration comparing the
stabilizing/destabilizing effects of charged residues at the e and g positions in homodimers
vs. heterodimers. Figure 3A shows a homodimer stabilized by oppositely-charged residues

10   at the e and g positions of a heptad. Figure 3B shows a heterodimer destabilized by
oppositely-charged residues at the e and g positions of a heptad. Figure 3C shows a
homodimer destabilized by positively-charged residues at the e and g positions of a heptad.
Figure 3D shows a heterodimer stabilized by like-charged residues at the e and g positions
of a heptad. Figure 3E shows a homodimer destabilized by negatively-charged residues at

15   the e and g positions of a heptad.

Figures 4A. 4B and 4C show a schematic of some possible distributions of heptads.
bearing either positive or negative charges at their e and g positions, within peptides
designed to form coiled-coil heterodimers. Figure 4A shows a schematic of a heterodimer
comprised of heterodimer-subunit peptides having alternating positively- and negatively-

20   charged successive heptads. Figure 4B shows a schematic of a heterodimer comprised of
heterodimer-subunit peptides. one of which has predominantly positively-charged heptads.
and the other of which has predominantly negatively-charged heptads. Figure 4C shows a
schematic of a heterodimer comprised of heterodimer-subunit peptides. one of which has all
positively-charged heptads, and the other of which has all negatively-charged heptads.

25   Figures 5A. 5B. 5C. 5D. 5E. 5F and 5G show a schematic of the synthesis of a
DNA fragment encoding a heterodimer-subunit peptide. Figure 5H shows a map of plasmid
pRLD-E.

Figure 6A shows an expression vector expression cassette useful for expressing a
fusion protein having a heterodimer-subunit peptide at the N-terminus of a selected protein

30   encoded by a DNA fragment inserted at the multiple cloning site (MCS).

Figure 6B shows an expression vector expression cassette useful for expressing a
fusion protein having a heterodimer-subunit peptide at the C-terminus of a selected protein
encoded by a DNA fragment inserted at the multiple cloning site (MCS).

Figure 7A shows a reversed-phase (RP)-HPLC chromatogram of peptide 0993 (SEQ

ID NO:26).

Figure 7B shows an RP-HPLC chromatogram of the injection flow through fraction of a selective dimerization affinity column of the invention.

Figure 8 shows an RP-HPLC chromatogram of the 60% acetonitrile wash fraction
5   of the affinity column described in Figure 7B.

Figure 9 shows an RP-HPLC chromatogram of a subsequent GndHCl elution fraction of the affinity column described in Figure 8.

Figure 10 shows a chromatogram of a mixture of three test charged peptides (SEQ ID NO:28, SEQ ID NO:29, and SEQ ID NO:30) used to test specificity of the affinity
10   matrix of the invention.

Figure 11 shows a chromatogram of the 0.2 M KCl wash fraction of an affinity column onto which test peptides depicted in Figure 10 were loaded.

Figure 12 shows a chromatogram of the 0.5 M KCl wash fraction of an affinity column to which test peptides were loaded.

15   Figure 13 shows a chromatogram of the 1.0 M KCl wash fraction of the affinity column described in Figure 12.

Figure 14 shows a chromatogram of a subsequent 1.0 M KCl wash of the column described in Figure 13.

Figure 15 shows a subsequent 5.0 M GndHCl/50mM K_2PO_4 of the column described
20   in Figure 14.

Figure 16A shows an SDS-PAGE gel of PAK(128-144)/E-coil fusion protein stained with comassie blue.

Figure 16B shows western and ligand blots of PAK(128-144)/E-coil fusion protein probed with either coil K reporter or monoclonal antibody PK99H.

25   Figure 17 shows a series of RP-HPLC chromatograms of load samples and fractions eluted from an affinity column of the invention, where the load fraction was a crude periplasmic extract containing recombinantly expressed E-coil peptide.

Figure 18 shows a series of RP-HPLC chromatograms of purification of recombinantly expressed Pak-pili-E-coil peptide from crude on an affinity column made in
30   accordance with the present invention.

Figure 19 shows an ELISA assay employing a reporter molecule formed according to the invention.

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Detailed Description f the Invention

I.   Definitions

       Unless otherwise indicated, the terms below have the following meaning:

       The term "peptide" designates a chain of amino acid based polyamides. The chain

5   can vary in length anywhere from 2 amino acids to about 100 amino acids.

       The term "polypeptide" designates a chain of amino acid based polyamides

containing at least 2 amino acids.

       A "promoter functional in a host" means a DNA sequence in an expression vector

effect to promote transcription of an adjacent 3' downstream coding sequence in the vector

10   in a selected host.

       A "coding segment" in an expression vector refers to a segment of DNA which

encodes a polypeptide.

       A "heterologous DNA coding site" refers to (i) a restriction site or sites, e.g.,

multiple cloning site, in an expression vector, into which a DNA that encodes a selected

15   heterologous protein can be inserted, or (ii) the encoding DNA itself.

       A "heterodimer-subunit peptide" refers to one of two complementary peptide

subunits which are capable of forming a coiled-coil heterodimer under selected conditions.

Further, the term "heterodimer polypeptide" or "heterodimer polypeptide complex" refers to

two associated non-identical polypeptide chains.

20   Unless otherwise indicated, the sequence for peptides and polypeptides is given in

the order from the amino terminus to the carboxyl terminus. All amino acid residues

identified herein are in the natural or L-configuration unless otherwise specified. In keeping

with standard peptide nomenclature, abbreviations for amino acid residues are standard 3-

letter and/or 1 letter codes commonly used in the art.

25   The term "derivatized", in the context of a "derivatized" polypeptide subunit, is

understood to refer to a polypeptide subunit having one or more functional or reporter

moieties covalently attached to one or more amino acid residues forming the subunit, where

the moiety may be (i) coupled to one.or more amino acid residues in the subunit either

before or after polypeptide subunit synthesis, or (ii) form an elongation of the peptide

30   subunit, e.g., at the subunit's N-terminus. Further, the functional or reporter moiety may

be attached to the polypeptide subunit directly, or through a linker or spacer, e.g., a poly-

glycine spacer.

       The term "benign medium" as used herein, describes a physiologically-compatible

aqueous solution typically having a pH of between about 6 and about 8 and a salt

concentration of between about 50 mM and about 500 mM. Preferably, the salt concentration is between about 100 mM and about 200 mM. An exemplary benign medium, designated as buffer A, has the following composition: 50 mM potassium phosphate, 100 mM KCl, pH 7. Equally effective benign media may be made by

5   substituting, for example, sodium phosphate for potassium phosphate and/or NaCl for KCl.

II. General Overview of Heterodimer-Subunit Peptides

Complementary heterodimer-subunit peptides are two non-identical peptide chains, typically about 21 to about 70 residues in length, having an amino acid sequence compatible

10   with their formation into two-stranded α-helical heterodimeric coiled-coils. They are designated herein generally as HSP1 (heterodimer-subunit peptide 1), and HSP2 (heterodimer-subunit peptide 2). Because the peptides can form α-helical coils, they are also referred to herein as "coil peptides".

In benign aqueous medium the isolated heterodimer-subunit peptides are typically

15   random coils. When HSP1 and HSP2 are mixed together under conditions favoring the formation of α-helical coiled-coil heterodimers, they interact to form a two-stranded α-helical coiled-coil heterodimeric complex, designated as HSP1 - HSP2.

Peptides in an α-helical coiled-coil conformation interact with one another in a characteristic manner that is determined by the primary sequence of each peptide: The

20   tertiary structure of an α-helix is such that 7 amino acid residues in the primary sequence correspond to approximately 2 turns of the α-helix. Accordingly, a primary amino acid sequence giving rise to an α-helical conformation may be broken down into units of 7 residues each, termed heptads. The heterodimer-subunit peptides are comprised of a series of heptads in tandem. When the sequence of a heptad is repeated in a particular

25   heterodimer-subunit peptide, the heptad may be referred to as a "heptad repeat", or simply "repeat".

As is detailed below, specific types of amino acid residues at defined positions in each heptad act to stabilize the two-stranded α-helical coiled-coil heterodimeric structure or complex.

30   HSP1 and HSP2 may also contain residues that can be reacted (either intra- or inter-helically) to stabilize the α-helical or coiled-coil nature of the polypeptides.

III. Features of Heterodimer-Subunit Peptides

Complementary heterodimer-subunit peptides (HSP1 and HSP2) are typically of

similar size, each generally ranging from about 21 to about 70 residues (3 to 10 heptads) in length.

The peptides may be synthesized by a variety of methods known to those skilled in the art, including chemical and recombinant methods. For example, an ABI Model 430A
5   peptide synthesizer may be used with conventional t-Boc chemistry as described previously by Hodges, *et al.*, (1988), and in Example 1. Alternatively, the peptides may be expressed in an appropriate host-cell system using an expression vector encoding the peptides, as detailed, *e.g.*, in Examples 5 and 6.

Subsequent to synthesis, the peptides may be purified by any of a number of
10   methods known to those skilled in the art, for example using reversed-phase high performance liquid chromatography (RPC) and a "SYNCHROPAK" RP-P column, as detailed in Example 1.

The composition and purity of the peptides can be verified by several methods, including amino acid composition mass analysis on a Beckman model 6300 amino acid
15   analyzer and molecular weight analysis using time of flight mass spectroscopy on a "BIOION-20" Nordic, as detailed in Example 1.

A.   Coiled-Coil Heterodimer Formation

The dimerization of HSP1 and HSP2 occurs due to the presence of a repeated
20   heptad motif of conserved amino acid residues in each peptide's primary amino acid sequence. The individual positions in each heptad are designated by the letters a through g for HSP1, and a' through g' for HSP2, as shown in Figure 2. The positions (*e.g.*, a', g') of HSP2 are sometimes referred to without the (') symbol in general discussions of heptad positions in heterodimer-subunit peptides, or coil peptides, below.

25   Repeating heptad motifs having appropriate amino acid sequences direct the HSP1 and HSP2 polypeptides to assemble into a heterodimeric α-helical coiled-coil structure under permissible conditions, presented in part D, below. The individual α-helical peptides contact one another along their respective hydrophobic faces, defined as the a and d positions of each heptad.

30   HSP1 and HSP2 may assemble into a heterodimer coiled-coil helix (coiled-coil heterodimer) in either parallel or antiparallel configurations. In a parallel configuration, the two heterodimer-subunit peptide helixes are aligned such that they have the same orientation (amino-terminal to carboxyl-terminal). In an antiparallel configuration, the helixes are arranged such that the amino-terminal end of one helix is aligned with the carboxyl-terminal

end of the other helix, and *vice versa*.

Diagrams of the relative orientations of the a-g positions of two interacting α-helices are shown in Figure 2. which shows an end-on schematic of the first two turns (one heptad) of two exemplary heterodimer-subunit peptides. 0993 (E: SEQ ID NO:26) and 0994
5 (K: SEQ ID NO:27) arranged in a parallel configuration.

Heterodimer-subunit peptides designed in accord with the guidance presented herein typically show a slight preference for assembling in a parallel orientation *vs.* an antiparallel orientation. Generally, however. the orientation (parallel *vs.* antiparallel) in which the two heterodimer-subunit peptides form an α-helical coiled coil is not particularly relevant to
10 their ability to hold together moieties attached to the heterodimer-subunit peptides.

In Figure 2. amino acids are circled and indicated by the one-letter code. and consecutive amino acid positions are numbered and joined by lines with arrow heads indicating the N-terminal to C-terminal direction. Interactions between the two helixes are indicated by arrows. Wide arrows crossing between the helixes depict hydrophobic
15 interactions between the a and d positions of adjacent helixes. Ionic interactions between the e and g positions of adjacent helixes are indicated as curving arrows above and below the nexus of the helixes.

B.   Hydrophobic Interactions in Coiled-Coil Heterodimer Stability

20   The hydrophobic interactions between the helixes are due to hydrophobic residues at the a and d positions of the heterodimer-subunit peptides. Residues at these positions. effective to maintain the helixes in contact. include leucine. isoleucine. valine. phenylalanine. methionine. tryptophan. tyrosine. alanine and derivatives of any of the above. Other residues, including alanine. cysteine, serine. threonine. asparagine and
25 glutamine may also occupy a or d positions in some heptads. so long as others are occupied by hydrophobic residues.

Appropriate selection of the specific residues to occupy the a and d positions is an important aspect of the present invention. If the hydrophobic interactions are too strong. a significant fraction of the helixes will form as homodimers at pH 7. even if like-charged
30 residues are present at the e and g positions to discourage homodimer formation (see part C., below). If, on the other hand, residues at the a and d positions are selected such that the hydrophobic interactions are too weak (for example. Ala at both positions). the helixes may not form coiled-coil dimers at all. Preferably. residue pairs at the a and d positions are selected to promote the formation $\geq$ 95% heterodimers at pH 7; more preferably $\geq$

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99% heterodimers. The degree of heterodimer *vs.* homodimer formation may be measured as described, for instance, in Example 3.

Results of experiments performed in support of the present invention indicate that if all the a and d positions in all heptads of both coil peptides are occupied by Ile. Leu. or

5   any combination thereof. the hydrophobic interactions will be strong enough to stabilize homodimers as well as heterodimers. even if the e and g positions contained charged residues designed to stabilize heterodimers as detailed herein. Further, such homodimers will form among coil peptides containing as few as three heptads. According to an important aspect of the invention, the formation of such homodimers should be minimized

10   or. for practical purposes, eliminated. The presence of a substantial fraction of homodimers would make practice of the invention difficult or impossible. since the coil peptides in the different component solutions (i.e., the coil peptides in a suspension containing fusions of a. selected protein and a coil peptide. or the coil peptides in a suspension to be used for derivatizing the solid matrix) would self-assemble before the component solutions could be

15   used for their intended purpose.

Several strategies may be employed to modulate or set the strength of hydrophobic interactions among residues at the a and d positions to a point where the interactions will be strong enough to maintain heterodimers during the protein purification procedure. yet not so strong that they will stabilize homodimers. An preferred strategy is to use coil peptides

20   where all heptads have Leu at position d. and Val at position a. or coil peptides where all heptads have Ile at position d. and Val at position a. Another strategy is to use to use coil peptides where all heptads have Leu at position a. and Val at position d. or coil peptides where all heptads have Ile at position a. and Val at position d. Variations of these strategies were employed in the design of exemplary heterodimer-subunit peptides E-coil

25   (SEQ ID NO:2) and K-coil (SEQ ID NO:4). as well as peptides 0993 (SEQ ID NO:26) and 0994 (SEQ ID NO:27). Yet another strategy is to occupy all but one of the a or d positions in each coil peptide by Ile and Leu. and use a destabilizing residue. such as Ala or Asn, in the remaining a or d position in each coil peptide. This remaining position is preferably closer to the center of each coil peptide than to either end.

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C.   Ionic Interactions in Coiled-coil Heterodimer Stability

Dimeric coiled-coil conformations of α-helixes can be stabilized by ionic interactions between residues at the e and g positions of adjacent helixes. as is illustrated in Figure 3. Other factors being equal, if each helix of a dimer has a positively-charged

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residue at ne position, for example, e, and a negatively-charged residue at the other position. for example. g. homodimer formation is favored (Fig. 3A; compare with heterodimer in Fig. 3B). However, if each helix has like-charged residues at both positions, then two oppositely-charged helices will tend to associate into heterodimers (Fig. 3D). as opposed to forming homodimers (Fig. 3C. 3E).

The conformation of polypeptides, such as HSP1 and HSP2. in solution can be determined from CD spectra of the solution. These data provide information as to the conformation of the individual peptides themselves (random coil vs. α-helical). as well information as to the relative amounts of heterodimer vs. homodimer complexes of. for example. HSP1 and HSP2. Example 2 details one method of measuring CD spectra. Example 3 details how a CD spectra measurements can be used to assess the conformation of peptides in solution.

In the diagram shown in Figure 2, ionic interactions between the two helixes arise from negatively-charged (Glu) residues at the e and g positions on HSP1 (0993; SEQ ID NO:26). and positively-charged (Lys) residues at the e and g positions on HSP2 (0994; SEQ ID NO:27).

Negatively-charged residues can be aspartic acid, glutamic acid or derivatives thereof. Positively-charged residues can be lysine. arginine. histidine. or derivatives thereof.

D.   Conditions Favorable for Coiled-coil Formation

Heterodimer-subunit peptides comprised of repeating heptads and designed according to the guidance presented in parts A through C. above. will readily form coiled-coil heterodimers in a benign medium, defined above in part 1. The degree of α-helical coiled-coil heterodimer formation can be determined from CD spectra, as described. for instance, in Example 3.

Coiled-coil heterodimers may form under conditions outside the pH and salt range given for a benign medium. but some of the molecular interactions and relative stability of heterodimers vs. homodimers may differ from characteristics detailed above. For example, ionic interactions between the e and g positions that tend to stabilize heterodimers may break down at low or high pH values due to the protonation of, for example, Glu side chains at acidic pH, or the deprotonation of, for example, Lys side chains at basic pH.

Aforementioned effects of low and high pH values on coiled-coil heterodimer formation may be overcome. however, by increasing salt concentration. Increasing the salt

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concentration can neutralize the stabilizing ionic attractions or suppress the destabilizing ionic repulsions. Certain salts have greater efficacy at neutralizing the ionic interactions. For example, a 1M or greater concentration of ClO_4^- anions may be sufficient to induce maximal α-helical structure (as determined by CD measurements performed as detailed in

5   Example 2), whereas a 3M or greater concentration of Cl^- ions may be required for the same effect. The effects of high salt on coiled-coil formation at low and high pH also show that interhelical ionic attractions are not essential for helix formation, but rather, are a factor in whether a coiled-coil tends to form as a heterodimer *vs.* a homodimer.

10   E.   Heptad Variation in Heterodimer-Subunit peptides.

Parts A, B and C, above, present guidelines as to which amino acid residues may be included, and which amino acid residues are preferable, at specific positions in heptads of heterodimer-subunit peptides that will typically result in those peptides forming α-helical coiled-coil structures in a benign medium. This part describes some examples of how

15   heptads with sequences which are in compliance with the guidelines presented in parts A through C, above, can be arranged within the heterodimer-subunit peptides.

Heterodimer-subunit peptides of the present invention may each contain from three to a plurality of heptads. The sequences of each of those heptads may all be the same, or they may differ. Furthermore, the sequences of the internal repeats may differ from one

20   another depending on, for example, whether or not the repeats incorporate amino acid coupling residues, such as cysteine. Such a coupling residue may be used, for example, to anchor a heterodimer-subunit peptides to a resin support matrix or to another polypeptide.

Because the salient interactions between two heterodimer-subunit peptides in an α-helical coiled-coil heterodimer pair are between adjacent, "complementary" heptads in each

25   peptide, the primary sequence of heptads within a heterodimer-subunit peptide can vary, so long as the residues within each heptad interact favorably with residues in the complementary heptad of the second heterodimer-subunit peptide.

It follows, then, that adjacent heptads may vary in sequence such that, for example, the net charge on the heterodimer-subunit peptides can be altered without affecting the

30   ability of the polypeptides to form α-helical heterodimer coiled-coils. This relationship is illustrated in Figure 4. The figure shows three examples of CP dimer pairs. Each heterodimer-subunit peptide has 5 heptads. The + or - symbols in each heptad each represent two charges (one at the e position and one at the g position). Note that adjacent complementary heptads have opposite charges. For the purpose of this example, it is

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assumed that positions other than e and g in each heptad sum to a net charge of zero. It can be appreciated that HSP1 and HSP2 forming the dimer in Figure 4A have net charges of +2 and -2, respectively. due to an excess of one positively-charged heptad. and one negatively-charged heptad. respectively. Similarly. HSP1 and HSP2 in Figure 4B have net

5   charges of +6 and -6, respectively. and HSP1 and HSP2 in Figure 4C have net charges of +10 and -10, respectively. Other variations on this theme are. of course, possible without departing from the spirit of the invention.

G.   Moiety Coupling to Heterodimer-Subunit Peptides

10   Coupling residues. such as amino-acid coupling residues. may be incorporated into one or both coil peptides to facilitate subsequent uses of the peptides. The coupling residues may be incorporated at in the central region of the peptide and/or at one or both ends. In cases where the coupling residues are in the central region, the residues are typically incorporated at positions b. c and/or f. preferably at position f, of one or more

15   heptads. These positions lie along the outward face of a coiled-coil heterodimer. Coupling residues at one or both ends are typically coupled to the terminal residue.

Preferred coupling groups are the thiol groups of cysteine residues, which are easily modified by standard methods. Example 4 details how the cysteine thiol groups present in coil peptides can be used to attach other peptides at those positions.

20   Other useful coupling groups include the thioester of methionine. the imidazolyl group of histidine. the guanidinyl group of arginine. the phenolic group of tyrosine and the indolyl group of tryptophan. These coupling groups can be derivatized in a manner similar to that detailed in Example 4. using reaction conditions known to those skilled in the art.

H.   Exemplary Coil Peptides

25   The E-coil (SEQ ID NO:2) and K-coil (SEQ ID NO:4) heterodimer-subunit peptides, as well as the 0993 (SEQ ID NO:26) and 0994 (SEQ ID NO:27), are exemplary HSP1 and HSP2 heterodimer-subunit peptides. Both peptides contain Val residues at their a positions, and Leu residues at their d positions. ensuring hydrophobic interactions effective

30   to stabilize coiled-coil heterodimers, but not strong enough to overcome the electrostatic repulsion between homodimers.

The e and g positions of 0993 heptads contain Glu residues. whereas the e and g positions of 0994 heptads contain Lys residues. The opposite charges at corresponding positions within complementary heptads of 0993 and 0994 stabilize α-helical coiled-coil

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heterodimers, as was described above and illustrated in Figs. 3A-E and 4.

In an analogous manner, the charged groups at the e and g positions discourage the formation of, and destabilize, homodimers. According to an aspect of the present invention, this destabilization is strong enough to overcome the hydrophobic interacti ns

5   present between appropriately-chosen residues at the a and d positions, which favor the formation of both heterodimers and homodimers.

As stated above, the salient interactions between two heterodimer-subunit peptides in an α-helical coiled-coil heterodimer pair are between adjacent, complementary heptads in each peptide. In a preferred embodiment of the invention, each of the corresponding d/a'

10   and a/d' pairs in such complementary heptads consists of residues where one of the residues is a valine and the other is selected from the group consisting of leucine and isoleucine. This relationship can be appreciated in Fig. 2, where the d/a' pair consists of Leu (at the d position) and Val (at the a' position), and the a/d' pair consists of Val (at the a position) and Leu (at the d' position).

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IV.   Protein Expression Vectors Employing Heterodimer-Subunit Peptides for Fusion
       Protein Purification

The invention includes an expression vector useful for expressing a fusion protein of a selected polypeptide expressed in tandem with a coil peptide (i.e, a heterodimer-subunit

20   peptide). The coil peptide portion of the expressed fusion may then be used to purify the protein using an affinity matrix (as described below), or to detect the protein, either *in situ* (*e.g.*, fluorescence detection in a cell) or in a protein detection system, such as enzyme-linked immunosorbent assay (ELISA), and/or a dot, slot or Western blot. The fusion protein is detected using a detection reagent which includes, *e.g.*, a reporter-labeled coil

25   peptide complementary to the coil peptide in the fusion.

Methods for cloning selected sequences into expression plasmids or vectors are known to those skilled in the art (*e.g.*, Maniatis, *et al.*, 1982; Ausubel, *et al.*, 1988). Such plasmids or vectors may then be transformed into suitable host cells, such as bacteria or yeast, and the cells induced to produce recombinant polypeptides. Depending on the

30   expression system, the medium in which the cells were induced, or the cells themselves, are used to make a suspension containing the recombinant polypeptide, which may then be purified using the methods of the invention.

By way of illustration, Example 5 details the recombinant construction of the E-coil gene using two sets of complementary overlapping oligonucleotides. The oligonucleotides

35   in each set were annealed, digested with the appropriate restriction enzymes to create sticky

ends at one end of each double-stranded (ds) fragment (Figs. 5C and 5F), and the two ds fragments were ligated with T4 DNA Ligase to generate a single ds fragment (Fig. 5G) of 177 base pairs. The 177 bp fragment was purified and digested with *EcoRl* and *BamHI* to produce a 166bp fragment suitable for cloning into a plasmid vector.

5 The construction of an exemplary plasmid expression vector, pRLD. is described in Example 6. pRLD was constructed by changing the polylinker site of expression vector pASK40 (Skerra. *et al.*, 1991) to correspond to the polylinker of pHIL-S1 and PIC9 (Invitrogen, San Diego, CA). This modification was designed to shift the reading frame and make pASK40's cloning site more consistent with that of *Pichia pastoris* vectors. A

10 sequence encoding the E-coil gene was then cloned into pRLD to produce the *E. coli* E-coil vector pRLD-E (Figure 5H).

The expression vector may contain a replication segment which permits autonomous replication of the vector in a selected host cell, and an expression cassette. The cassette contains, in a 5'-3' direction. a promoter functional in the host cell and a coding segment,

15 which is described in detail. below. The vector may also contain additional elements. such as sequences encoding a selectable marker that assure maintenance of the vector in the cell. an appropriately positioned ribosome-binding site downstream of the promoter. and transcription termination (TT) sequences at the 3' end of the cassette.

The elements preferably contained in expression vectors depend on the particular expression system with which the vectors are used. For example. elements typically present

20 in *E. coli* expression vectors include (i) sequences encoding a selectable marker that assure maintenance of the vector in the cell (*e.g.*, an ampicillin resistance gene). (ii) a controllable transcriptional promoter which. upon induction, can produce large amounts of mRNA from a selected gene cloned into the vector, (iii) translational control sequences, such as an

25 appropriately positioned ribosome-binding site and initiator ATG. and (iv) a polylinker or multiple cloning site (MCS) to simplify the insertion of the selected gene in the correct orientation within the vector.

Examples of inducible promoters suitable for use with *E. coli* expression vectors include *lac*, *trp*, and *tac*, which, upon induction, can produce large amounts of mRNA from

30 genes operatively linked to the promoters. Other promoters suitable for use with *E. coli* expression vectors include the highly-efficient phage T7 gene 10 promoter, which uses T7 RNA polymerase, and the powerful bacteriophage pL promoter (Ausubel, *et al.*, 1988).

Recombinant polypeptides or fusion proteins are typically expressed by transforming a suitable *E. coli* expression strain (*e.g.*, JM83) with expression plasmids encoding

recombinant polypeptides or fusion proteins. Examples of suitable transformation methods include heat-shock treatment (Yanisch-Perron. *et al.*, 1985) and electroporation. The transformed cells are then typically plated on selective agar plates (*e.g.*, LB plates supplemented with carbenicillin (100 μg/ml)), and cells c ntaining the appropriate

5   recombinant DNA are verified by restriction digest analysis and subsequent DNA sequencing of minipreped plasmid DNA.

Selected *E. coli* clonal cells harboring the appropriate DNA are typically grown (e.g., at 25°C with shaking in LB medium containing 100 μg/μl of carbenicillin) to a suitable density (e.g., an A$_{550}$ of 0.5). When using plasmids requiring induction, *e.g.*, with

10   IPTG, the cells are induced by addition of the appropriate inducing agent.

A suspension containing the expressed protein may be obtained using methods known in the art. e.g., a modified osmotic shock treatment (Ausubel, *et al.*, 1988). Although proteins expressed in large amounts in *E. coli* sometimes precipitate into insoluble aggregates (inclusion bodies). such proteins may be recovered using, for example,

15   solubilization in denaturing agents known to those skilled in the art (Ausubel. *et al.*, 1988).

Other types of host cell expression systems may be employed in the practice of the invention. For example, yeast, such as *Pichia pastoris*, offers a number of advantages, including very high expression levels, ease of use and some postranslational modification. Yeast expression vectors may be used to transform yeast spheroplasts, and the transformed

20   cells used to produce recombinant polypeptide. Exemplary inducible promoters suitable for use with yeast expression vectors are the AOX promoter, described above, and the *GAL1*, *GAL4*, *GAL7* and the *GAL10* promoters. Example 7 describes the use of a yeast expression vector in the practice of the invention. A DNA fragment containing the E-coil gene was PCR-amplified and cloned into plasmid pIC9 (Invitrogen. San Diego. CA), generating

25   *Pichia* E-coil vector p9CE. Verified yeast plasmids were transformed into *Pichia pastoris*, and the yeast cells were selected, induced and processed as described in Example 7 to generate culture supernatant containing the recombinant protein.

Expression cassettes or coding segments of the present invention may also be used to express fusion proteins using the baculovirus system, where genes for proteins to be

30   expressed are inserted into an insect virus in lieu of a highly expressed dispensable gene. The foreign protein is produced by growing the recombinant virus in cultured insect cells (Ausubel, *et al.*, 1988). Such vectors typically contain recombination sequences to allow the vector to integrate into the host cell's genome via homologous recombination. The specific promoters used in these vectors generally depend on the identity of the excised

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dispensable gene. Exemplary promoters include the polyhedrin promoter (normally used to drive expression of the AcMNPV polyhedrin gene) and the p10 promoter (Ausubel, *et al.*, 1988).

5   Mammalian cells may also be used as host cells for vectors employing expression cassettes or coding segments of the present invention. Two exemplary mammalian systems employ the COS and CHO cell lines, respectively. In the COS system, vectors containing the gene to be expressed are transiently transfected into COS cells, which constitutively produce SV40 large T antigen. Vectors used with COS cells typically contain an SV40 replication origin, such that when the vectors are transfected into COS cells, they replicate

10   and thereby amplify the amount of protein expressed.

       CHO cells may be stably transfected with vectors employing expression cassettes or coding segments of the present invention and carrying either the dihydrofolate reductase or the glutamine synthetase gene (whose products confer drug resistance). Cell lines that carry increased numbers of the constructs are obtained by selecting those that grow in increasing

15   drug concentrations (e.g., methotrexate). Once selected, these lines are permanent reagents, which can be stored frozen and used to produce the protein whenever desired.

Coding Segment

       The coding segment contains a heterologous DNA coding site, a DNA region

20   encoding a first heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a complementary second heterodimer-subunit peptide, and disposed between the coding site and the DNA region, a cleavage sequence. The cleavage sequence is in frame with the DNA region, and encodes an amino acid sequence that provides a target for chemical or enzymatic cleavage. The cleavage sequence is preferably included in

25   coding segments of the present invention to facilitate the removal of the coil peptide from its fusion partner (the selected polypeptide of interest). However, it will be understood that in applications where it is not desired to remove the coil peptide from the fusion protein, the cleavage sequence is not necessary. In this respect, the invention contemplates a coding segment containing only a heterologous DNA coding site and a DNA region encoding a

30   first heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a complementary second heterodimer-subunit peptide.

       In a general embodiment, the coding site is a multiple cloning site (MCS), also referred to as a polylinker or polylinker site. The MCS typically contains a number of restriction enzyme sites useful for cloning selected polynucleotides to be expressed into the

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coding segment or vector containing the coding segment. Examples of suitable polylinkers
can be found in virtually all commercially-available cloning vectors (e.g.. vectors from
Stratagene. La Jolla, CA or Invitrogen. San Diego, CA).

In other embodiments. the coding site contains a gene encoding a protein of interest.
5   The generation and incorporation of such genes into expression vectors of the invention is
described in Examples 9 and 10. respectively. In Example 9. a gene encoding PAK pili
antigen is generated by annealing synthetically-produced complementary oligonucleotides
and cloning the ds fragment into appropriate restriction sites in the pRLDE vector. In
Example 10. a cDNA encoding GFP is cloned into pRLDE. Exemplary proteins which
10 may be purified using the methods of the present invention include epoetin α, G-CSF,
somatotropic. insulin, tPA, urokinase and prourokinase. various interferons, EPO. and
various specialty enzymes.

The coding segment may contain the coding site oriented either 5' or 3' of DNA
region. These two possibilities are illustrated Figs. 6A and 6B. which show schematics of
15 expression cassettes of the present invention. The cassettes contain a promoter functional in
the selected host cell system. and, disposed at the 3' ends of the promoter regions. coding
segments as described above. In Figs. 6A and 6B. the heterologous DNA coding sites are
represented as "cDNA" and the DNA regions encoding a first heterodimer-subunit peptide
are represented as "Coil DNA". Figure 6A shows the coding site oriented at the 3' end of
20 the DNA region. whereas Figure 6B shows the coding site oriented at the 5' end of the
DNA region.

Cleavage sites that constitute targets for rare-cutting proteases are known in the art
(Ausubel. et al., 1988). Such proteases include factor Xa (Nagai and Thogersen. 1984.
1987). thrombin (Smith and Johnson. 1988: Gearing. et al., 1989). enterokinase (Dykes. et
25 al., 1988: LaVallie. et al., 1993). renin (Haffey. et al., 1987). and collagenase (Germino
and Bastia, 1984). Factor Xa and enterokinase are particularly useful because they cleave
on the carboxy-terminal side of their respective recognition sequences, allowing the release
of fusion partners containing their authentic amino-termini. The recognition sites for
enterokinase. factor Xa and thrombin are provided herein as SEQ ID NO:21, SEQ ID
30 NO:23 and SEQ ID NO:25. respectively.

The primary reason for providing coding segments having the two different
arrangements of elements described above is that fusions of a peptide (such as a coil
peptide) with a selected polypeptide of interest sometimes alter a desired activity or
characteristic of the polypeptide of interest. By generating two different fusion, one of

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which has the coil peptide at the N-terminus, and the other of which has the coil peptide at the C-terminus of the selected polypeptide of interest. the probability of obtaining a fusion that does not significantly alter a desired property or characteristic of the selected polypeptide of interest is increased.

5   Exemplary coding sequences for the DNA region encoding the first heterodimer-subunit peptide are the sequences represented by SEQ ID NO:2. SEQ ID NO:4. SEQ ID NO:26 and SEQ ID NO:27. It will be appreciated that in the case illustrated in Fig. 6A. where the DNA region is upstream of the coding site. the DNA region typically includes an initiation ATG codon as its first codon. In cases such as illustrated in Fig. 6B. where the

10   DNA region is downstream of the coding site, the initiation ATG codon is typically provided by the DNA encoding the polypeptide of interest inserted at the coding site.

Coding segments of the present invention are preferably provided in three configurations for each of the two arrangements of elements described above. The three forms each differ from one another by having different-length nucleotide spacers between

15   the coding site and the cleavage site. The spacers are included to provide coding segments having the coding site in all three reading frames relative to the reading frame of the DNA region and the cleavage site. In this way, an appropriate coding segment can be selected by those of skill in the art to express any DNA fragment having ends compatible with restriction sites present at the MCS in frame with the DNA region and the cleavage site.

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V.   Affinity Matrix Composition Employing Heterodimer-Subunit Peptides for Fusion
      Protein Purification

The invention also includes an affinity matrix composition useful for purifying polypeptides, such as fusion proteins containing selected polypeptides expressed in tandem

25   with a first heterodimer-subunit peptide. The matrix includes a solid support having attached thereto a heterodimer-subunit peptide that is capable of forming an α-helical coiled-coil with another, complementary heterodimer-subunit peptide expressed as part of a fusion protein. The affinity feature of the matrix is based on selective dimerization of the complementary heterodimer-subunit peptides.

30   The selective dimerization process for purification is shown schematically in Figures 1A-1F. Figure 1A shows a selective dimerization matrix 30 consisting of solid phase matrix 32 and heterodimer-subunit peptide 34. Optionally matrix 32 is conjugated to peptide 34 through a spacing group 36. The presence of such a group may be particularly advantageous when the protein to be purified on the column is a large or sterically bulky

35   protein.

Solid phase matrix 32 may be formed by any of a number of suitable materials known in the art. Preferably, the solid phase material will consist of spherical particles, such as aminopropyl-derivatized glass particles, capable of forming a packed matrix in a chromatography column; however, the solid phase may be formed by a continuous surface

5   or by non-spherical particles or beads. The method used for conjugation of the peptide to the solid phase will be readily determined by the skilled practitioner, based on the type of matrix, the type of spacing group, if present, and the desired peptide linkage.

The fusion protein is purified by first obtaining, from the host cell expression system, a suspension containing the fusion protein using methods known in the art (e.g.,

10   Ausubel, et al.). This suspension is typically an impure solution containing impurities, such as other proteins, in addition to the desired fusion protein.

With reference to Figure 1B, for purification of such a fusion protein from an impure solution containing other proteins, it can be seen that as the suspension is passed over the affinity matrix composition, the affinity matrix 30 binds to fusion protein 38

15   containing selected polypeptide 40, and heterodimer-subunit peptide 42, complementary to the immobilized heterodimer-subunit peptide 34. Also shown are non-binding components of the solution such as impurities 44 that do not specifically interact with the heterodimer-subunit peptide on the column. These impurities are removed from the column milieu by extensive washing of the column.

20   Exemplary wash conditions include a salt wash step with a solution having a pH of between about 5.5 and about 8.0 (e.g., 6.0) and containing between about 0.1 M and about 1 M (e.g., 0.5 M) salt (e.g., NaCl), and an organic wash step with a solution having a pH of between about 5.5 and 8.0 (e.g., 6.0) and containing between about 50% and about 100% (e.g., 75%) of a suitable organic solvent (e.g., acetonitrile). Suitable salts include

25   sodium chloride (NaCl), potassium chloride (KCl), sodium acetate (NaOAc), ammonium chloride (NH$_4$Cl), ammonium acetate (NH$_4$OAc), sodium perchlorate (NaOClO$_4$), potassium perchlorate (KOClO$_4$), sodium phosphate (Na$_2$HPO$_4$), and potassium phosphate (K$_2$HPO$_4$). Suitable organic solvents include methanol (MeOH), ethanol (EtOH), isopropanol, tetrahydrofuran (THF), trifluoroethanol (TFE), and acetonitrile.

30   ALthough the order in which the salt and organic washes are performed is not critical, if the organic wash is done first, the column should be rinsed well with an aqueous solution (e.g., water) before the start of the salt wash, to remove as much of the residual organic solvent as possible. For this reason, it is generally preferable to do the salt wash first, followed by the organic wash, followed by a final aqueous rinse before the desired

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protein is eluted from the column.

Figure 1C shows a schematic of the column matrix after a wash step. Impurities shown in Figure 1B are now absent from the column milieu. Fusion proteins 38 are now bound to the column through an interaction between immobilized heterodimer-subunit peptide 34 and complementary subunit 42 that forms a part of fusion protein 38.

Figures 1D and 1E-F show alternative means of eluting selected protein 40 from the column (i.e., means for releasing the selected polypeptide from the matrix). Figure 1E depicts a scheme in which the fusion polypeptide 38 is eluted from the column by washing with a buffer, such as a highly ionic buffer, that disrupts the interactions between the subunits of the coiled-coil heterodimer. As illustrated, fusion protein 38 has been removed from the column by contact with such a buffer. As is detailed below, a suitable ionic buffer is 0.5 M guanidium chloride. Alternatively, the fusion protein may be removed by eluting with a solution having a pH of less than about 3.0 and containing between about 20% and about 80% (e.g., 50%) of a suitable organic solvent (e.g., acetonitrile). The low pH of such an elution solution results in protonation of the negatively-charged residues (e.g., Glu), disrupting the ionic interactions that stabilize the heterodimer. In another embodiment, the fusion protein is eluted with a solution having a pH of more than about 10.0 and containing between about 20% and about 80% (e.g., 50%) of a suitable organic solvent (e.g., acetonitrile). The high pH of such an elution solution results in neutralization of the positively-charged residues (e.g., Lys), disrupting the ionic interactions that stabilize the heterodimer.

To complete the purification process, selected polypeptide 40 may be cleaved (Fig 1F) from complementary heterodimer-subunit peptide 42 by a peptide cleavage reaction, such as by enzymatic cleavage.

As an alternative, selected polypeptide 40 is directly removed from the column by cleavage in situ, as by enzymatic cleavage. As illustrated in Figure 1D, following cleavage, the polypeptide divides into complementary subunit portion 42, which remains bound as a coiled-coil heterodimer to column matrix heterodimer-subunit peptide 34, and selected protein or polypeptide 40.

From the foregoing it will be seen that the selective dimerization method of the invention is a useful and efficient means for purifying proteins, particularly fusion proteins, containing a heterodimer-subunit peptide capable of forming an α-helical coiled-coil in accordance with the present invention.

Examples 8 and 12 describe the preparation and use of an affinity matrix designed

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for purification of polypeptides containing a coil peptide. including exemplary coupling protocols to attach one of complementary heterodimer-subunit peptides to the matrix. preparation of an affinity column using the derivatized matrix, analysis of complementary heterodimer-subunit peptides and/or suspensions containing various contaminants along with

5   such peptides as fusions with selected proteins run through such a column, and removal of the selected peptides and/or fusions from the column.

Example 11 describes results of Western and ligand blot analyses of proteins purified using the above-described method. Figure 16A shows an image of a gel stained with Coomassie blue showing the relative purity of PAK (128-144)/E-coil purified using a

10   K-coil affinity column. The gel was blotted and subjected to Western blot and ligand blot analyses as described in the Example. The results, shown in Figure 16B, show that methods of the invention can be successfully used to effectively purify recombinant fusion proteins or polypeptides.

15   VI.   Detection Reagents Employing Heterodimer-Subunit Peptides

The invention includes synthesis and use of a reagent for detecting expressed proteins. in particular fusion proteins having a heterodimer-subunit peptide capable of forming an α-helical subunit in conformance with the invention. The reagent can be used in a number of research and clinical applications. including. but not limited to detection of

20   expression of fusion proteins. ELISA applications. radioimmunoassays and the like. The detection reagent may also form part of a kit for detecting the expression of a selected polypeptide. where the kit also includes an expression vector that includes a DNA region encoding a heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer in accordance with the present invention.

25   In one form, the detection reagent includes (i) a heterodimer-subunit peptide designed and formed in accordance with the methods described herein, and (ii) a reporter molecule. The reporter molecule may be coupled to the heterodimer subunit peptide (e.g., coupled to an amino acid coupling residue, such as a cysteine residue, at an outwardly-facing heptad position (e.g., the f position) of the heterodimer-subunit peptide, or it may be

30   synthesized. for example, in tandem with the coil peptide (e.g., as described in Example 10).

Alternatively reporter molecule may be incorporated into the heterodimer subunit. for example as a radioactive amino acid. Radioactive amino acids are commercially available from a number of sources and can be introduced into the heterodimer-subunit

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peptide during chemical or biological synthesis. according to methods well known in the
art.

The reporter molecule can be selected from a number of reporter molecules well
known in the art. including but not limited to radioactive groups. fluorescent tags. enzymes
5   and the like. Use of enzyme markers and methods of incorporating them into biological
detection systems are known in the art (see. for example. Ausubel. *et al.* (1988). for
general methods: for specific exemplary reporter enzymes. see. for example. the following:
luciferase (Brasier. *et al.*, 1989: Gould and Subramani. 1988). chloramphenicol
acetyltransferase (CAT: Gorman. *et al.*, 1982). β-galactosidase (An, *et al.*, 1982)).
10   Preferably. the reporter molecule is attached to a coupling position in the heterodimer-
subunit peptide that does not interfere with heterodimer formation. One preferred position
is position f of the subunit.

Example 13 provides details for forming a heterodimer-subunit peptide reporter
molecule in accordance with the invention. Figure 19 depicts a reporter molecule in a solid
15   phase (ELISA) assay in a specific embodiment used to detect the presence of expressed
proteins in accordance with the present invention.

In one representative embodiment. the reporter molecule is employed in an ELISA
detection system used to quantitatively detect the presence of a heterodimer-subunit fusion
protein. such as the PAK pili antigen/E-coil fusion protein described in Example 9.
20   Figure 19 shows a schematic diagram of an ELISA detection system to detect the a
fusion protein formed from a 17 amino acid *Pseudomonas aeruginosa* strain PAK pilin-
derived peptide. Referring to the figure. a solid phase such as plate 50 is coated with a
capture molecule such as PAK pilin peptide-specific antibody PK99H 52. according to
standard methods known in the art. A sample containing fusion protein 54 is contacted with
25   the plate under conditions that promote binding of the protein to antibody 52. As illustrated
fusion protein 54 is composed of a heterodimer-subunit peptide 56 and PAK pilin peptide
58. Following washing of the plate to remove unbound proteins. detection reagent 60,
composed of a complementary heterodimer-subunit peptide 62 attached to reporter molecule
64, is added to the plate and allowed to react. under conditions that promote formation of a
30   coiled-coil heterodimer complex between the complementary heterodimer-subunit peptides
56 and 62. Following further washing of the plate. detection of reporter molecule is used
to quantitate the amount of fusion protein 54 bound to the plate.

Other embodiments of the invention using the reporter molecule will be apparent
from the description provided above. For example. the reagent can be used to detect the

26

presence of a complementary heterodimer-subunit peptide-containing fusi n protein in a gel
or on a blot: likewise the reagent can be used to detect expression of the fusion protein by
plaques or in cell expression systems. according to methods known in the art.

5   The following examples illustrate. but in no way are intended to limit the present
invention.

Materials and Methods

A.   Abbreviations

10   Abbreviations used in the Examples are t-BOC. tertiary butoxycarbonyl; DCM,
dichloromethane; DIEA. diisopropylethylamine; DCC. dicyclohexylcarbodimide; DMF.
N.N-dimethylformamide: Gnd. guanidinium; HF. hydrogen fluoride: DCU.
dicyclohexylurea: BSA. bovine serum albumin.

15   ### B.   Buffers

Phosphate-buffered saline (PBS)

10x stock solution. 1 liter:
80 g NaCl
2 g KCl
20   11.5 g $Na_2HPO4-7H_2O$
2 g KH_2PO_4

Working solution. pH 7.3:
137 mM NaCl
25   2.7 mM KCl
4.3 mM $Na_2HPO_4-7H_2O$
1.4 mM KH_2PO_4

C.   Strains and Plasmids

30   *E. coli* expression strain JM83 (Yanisch-Perron, *et al.*, 1985) obtained from (New
England Biolabs, Beverly, MA) was used as the host cell in experiments exemplifying a
prokaryotic expression system. Cells were cultured in Luria broth (LB) media (Miller,
1972) supplemented with 100 μg/ml of carbenicillin. Plasmids for the eukaryotic system
were propagated and cloned in *E. coli* strain Top10F' (Grant, *et al.*, 1990) and expressed in
35   *Pichia pastoris* strain GS115 (Cregg, *et al.*, 1985), both purchased from Invitrogen (San
Diego, CA).

Plasmid pASK40 (Skerra, *et al.*, 1991) was used for plasmid construction for *E.
coli* expression. Yeast expression plasmid pIC-9 (Invitrogen) was used for plasmid

construction for yeast expression.

D.   Enzymes and Oligonucleotides

Restriction enzymes and DNA modifying enzymes were purchased from GIBCO

5   BRL (Gaithersburg, MD). Deoxyribonucleotides were prepared on an automatic DNA

synthesizer Model 380A (Perkin-Elmer Applied Biosystems Division, Foster City, CA).

Isolation of plasmid DNA and routine manipulations were carried out according to standard

procedures (Sambrook, et al., 1989; Ausubel, et al., 1988). Dideoxy-sequencing (Maxam

and Gilbert, 1977) of the cloned plasmid constructs was performed using a modified

10   polymerase chain reaction (PCR) cycle sequencing protocol (Craxton, 1991) employing

biotinalated primers and chemiluminescent detection (Amersham, Arlington Heights, IL).

E.   SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

15   performed with 15% acrylamide gels in a mini-gel apparatus ("MINI-PROTEIN II" Dual

Slab Cell, Bio-Rad Laboratories, Hercules, CA) as described by Laemmli, et al. (1973).

Gels were stained with Coomassie blue (R-250, Bio-Rad).

F.   Western Blot

20   Proteins on the SDS-PAGE gel were transferred to nitrocellulose membrane using

the protocol of Towbin, et al. (1979), with a Mini Trans-Blot Electrophoretic Transfer Cell

(Bio-Rad Laboratories). The transfer was typically completed after 30 min under constant

current of 300 mA (Model 200/2.0 Power supply, BioRad).

Excess binding sites on the membrane were blocked by incubation of the blots with

25   a blocking solution consisting of 50 mM tris-hydroxy-methyl aminomethane (Tris) HCl, pH

7.5, 150 mM NaCl, 0.05% (v/v) "NONIDET-P40", 0.25% (w/v) gelatin and 3% (w/v)

BSA at room temperature for 1 hr in an incubator shaker (model G25 Gyroshaker, New

Brunswick Scientific, New Jersey, USA) set at 100 rpm. The membrane was washed twice

at room temperature with 10 mM Tris-HCl buffer pH 7.5 containing 0.1% "TWEEN-20"

30   and 0.05% (w/v) BSA (TTBS).

G.   Ligand Blot

Proteins resolved on an SDS-PAGE gel were transferred to nitrocellulose membrane

as described above. The proteins on the membrane were denatured by soaking the

membrane for 1 hr in PBS-ED buffer (20 mM potassium phosphate, pH 7.9, 200 mM KCl, 1 mM EDTA, 1 mM DTT) containing 6 M guanidine HCl. The proteins were gradually renatured by four serial 1:1 dilutions of the membrane in PBS-ED and washed free f the residual guanidine HCl in fresh PBS-ED buffer. The membrane was blocked for 1 hr in

5   PBS-ED blocking buffer containing 0.05% (v/v) Nonidet-P40, 0.25% (w/v) gelatin and 3% (w/v) BSA at room temperature for 1 hr in an incubator shaker. The blots were rinsed once with PBS-ED.

_____

EXAMPLE 1

10 ### Chemical Synthesis, Purification and Analysis

Peptides were chemically-synthesized by solid-phase peptide synthesis using a benzhydryl amine-hydrochloride resin on an Applied Biosystems (Foster City, CA) peptide synthesizer Model 430A with conventional N-t-butyloxycarbonyl (t-Boc) chemistry as described previously (Hodges, et al., 1988). The peptides were cleaved from the resin by

15 reaction with hydrofluoric acid (HF; 20 ml/g resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 hour at -5°C to 0°C.

The crude reduced peptides were purified by reversed-phase high performance liquid chromatography (RPC) and a "SYNCHROPAK" RP-P semi-preparative C_{18} column (250 × 10 mm inner diameter, 6.5 μm particle size, 300 Å pore size: SynChrom,

20 Lafayette, IN) with a linear AB gradient of 0.5% B/min and 2 ml/min, where solvent A was 0.05% trifluoroacetic acid (TFA) in water and solvent B was 0.05% TFA in acetonitrile.

The amino acid composition and mass analysis were consistent with the designed sequence. For amino acid analysis, purified peptides were hydrolyzed in 6 N HCl

25 containing 0.1% phenol at 100°C for 24 hours or 1 hour at 160°C in evacuated sealed tubes. Amino acid analysis was performed on a Beckman model 6300 amino acid analyzer (Beckman, San Ramon, CA). The correct primary ion molecular weights of the reduced peptides were confirmed by plasma desorption time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

30

EXAMPLE 2

Circular Dichroism Measurements

Circular dichroism (CD) spectra were recorded at 20°C on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco DP-500N data processor and

a Lauda (model RMS) water bath (Brinkmann Instruments. Rexdale. Ontario. Canada) for

control of the temperature of the cuvette. Constant N$_2$ flushing was employed. The

instrument was routinely calibrated with an aqueous solution of recrystallized d-10-(+)-

camphorsulfonic acid at 290 nm.

5   Molar ellipticity at 200 nm is reported as mean residue molar ellipticity ([θ]$_{220}$.

deg•cm^2•dmol^{-1}) and calculated from the equation:

$$[\theta] = [\theta]_{obs} \times mrw/10 \times l \times c$$

10   [θ]$_{obs}$ is the ellipticity measured in degrees. mrw is the mean residue molecular weight

(molecular weight of the peptide divided by the number of amino acid residues). c is the

peptide concentration in grams per milliliter. and l is the optical path length of the cell in

centimeters. CD spectra were the average of four scans obtained by collecting data at 0.1-

nm intervals from 250 to 190 nm.

15   Peptide concentrations were determined by amino acid analysis. The pH was

measured at room temperature.

EXAMPLE 3

Heterodimer vs. Homodimer Formation

20   Peptides 0993 (SEQ ID NO:26) and 0994 (SEQ ID NO:27) were synthesized as

described in Example 1. CD spectra of peptide mixtures of different ratios of the first

heterodimer-subunit peptide 0993 (SEQ ID NO:26) and the second heterodimer-subunit

peptide 0994 (SEQ ID NO:27) were measured as described in Example 2. to determine the

degree of heterodimer vs. homodimer formation.

25   The peptides were suspended in a solution containing 0.1 M KCl and 50 mM

potassium phosphate buffer, pH 7 at 20°C (reaction buffer). The total peptide

concentration (sum of 0993 and 0994 concentrations) was 20 μM.

The data show that as the ratio of the peptides was changed from 0:100 to 50:50,

the conformation of the peptide mixture changed from a random coil structure to an α-

30   helical structure. An equimolar mixture of the 0993 and 0994 peptides displayed the double

minima at 220 and 208 nm with -31,760 deg•cm^2•dmol^{-1} of mean residue ellipticity at 220

nm. which corresponds to ~ 100% α-helical structure (Hodges, et al., 1990), suggesting

that the interhelical ionic repulsions which destabilize the homo-stranded coiled-coil provide

a driving force for the formation of the hetero-stranded coiled-coil.

These results indicate that the mixture of peptides 0993 and 0994 forms a hetero-stranded coiled-coil.

<div align="center">

EXAMPLE 4

Linking Peptides to Heterodimer Scaffold
by Alkylation of Thiol Groups

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Conjugation to peptide sulphydryl groups was carried out at ambient temperature in 50 mM NH$_4$OAc and 8 M urea at pH 8. Bromo- or iodacetylated peptides were dissolved in buffer (0.987 μM, 2 ml) and peptide 0994 (SEQ ID NO:27) was added to a final concentration of 0.165 μM (2 ml). The reaction mixture remained clear and was allowed to react at ambient temperature for 22 hours, at which time it was acidified by the careful addition of TFA (pH 2) and lyophilized.

A.   Conjugate Purification and Identification

The reaction mixture (2 ml) was applied directly to a Synchropak RP-8 semi-prep column (250 mm × 10 mm I.D.; Synchrom Inc., Lafayette, IN). The conjugate was easily separated from unreacted peptide using gradient elution (2% B/minute over 30 minutes. Solvent A: 0.05% TFA/H$_2$O; Solvent B: 0.05% TFA/acetonitrile). The isolated conjugate was lyophilized and redissolved in HPLC grade water (200 μl) which was then applied to a Mono-S strong cation exchange column (Pharmacia, Uppsala, Sweden) for further purification. The gradient employed during this purification step was a 1% B/minute gradient (Solvent A: 5 mM NaH$_2$PO$_4$/20% acetonitrile, pH 5. Solvent B: 5 mM NaH$_2$PO$_4$/20% acetonitrile, 1 M NaCl, pH 5). The isolated conjugate was then desalted using a reversed-phase column and a standard 2% B gradient (vide supra). In this way, pure conjugate was obtained which was shown through mass spectrometric analysis to be the desired product (MW calc: 7432.0, Found, 7432.4).

<div align="center">

EXAMPLE 5

Recombinant Coil Gene Synthesis

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The E-coil gene was synthesized as follows. A DNA sequence was constructed from back translation of the de novo designed coil protein sequence using a codon bias based the on highly expressed Pichia pastoris genes (Koutz, et al., 1989). To avoid creation of excess restriction sites, four overlapping oligonucleotides were synthesized and purified by electroelution on a 12% polyacrylamide gel containing 7M urea. The coil DNA fragments were synthesized using Klenow fragment of E. coli DNA Polymerase 1 as shown

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in Figs. 5A through 5G.

Oligonucleotides 1 (SEQ ID NO:6) and 2 (SEQ ID NO:7) were annealed together by their complementary sequences and the remaining complementary strands were synthesized by Klenow fragment as illustrated in Figures 5A and 5B. An identical protocol

5   was carried out for oligonucleotides 3 (SEQ ID NO:8) and 4 (SEQ ID NO:9) (Figs. 5D and 5E).

The double stranded fragments were then digested with restriction enzyme *Alw21* I to create sticky ends at one end of each fragment (Figs. 5C and 5F), and the two fragments were ligated with T4 DNA Ligase to generate a double-stranded fragment (Fig. 5G) of 177

10   base pairs. The fragment was excised, electroeluted from the gel slice, and digested simultaneously with *EcoRI* and *BamHI* to produce a 166bp fragment suitable for cloning into a plasmid vector.

EXAMPLE 6

15   ### Expression of Coil Constructs in *E. coli*

Expression vector pASK40 was modified at its polycloning site to produce plasmid pRLD. pASK40 was digested with *EcoRI* and the resulting 5' protruding ends were blunted with Mung-Bean Nuclease. The blunted plasmid was then digested with *Hind*III and religated with a synthetic 43 bp annealed deoxyribonucleotide fragment formed by

20   annealing oligonucleotides 5 (SEQ ID NO:10) and 6 (SEQ ID NO:11). This modification. which was verified by double stranded DNA sequencing, was designed to shift the reading frame and make pASK40's cloning site more consistent with that of *Pichia pastoris* vectors.

The synthesized E-coil gene was double digested with *EcoRI* and *BamHI* as described above and force-cloned into pRLD digested with *EcoRI* and *BglII* to produce the

25   *E. coli* E-coil vector pRLD-E (Figure 5H). The ligated plasmids were transformed into *E. coli* expression strain JM83 by heat-shock treatment (Yanisch-Perron, *et al.*, 1985) and plated on selective LB plates supplemented with carbenicillin (100 µg/ml). Insertion of the coil gene was verified by restriction digest analysis and subsequent DNA sequencing of minipreped plasmid DNA of successful transformants.

30   *E. coli* cells harboring pRLD-E were grown at 25°C with shaking in LB medium containing 100 µg/µl of carbenicillin to an A_{550} of 0.5. Expression was induced by adding isopropylthiogalactoside (IPTG) to a final concentration of 1mM and incubating for 3 hours at 25°C with shaking.

Expressed protein was obtained by a modified osmotic shock treatment (Ausubel, *et*

32

_al.._ 1988). Cells were collected at 4000 ×_g_ for 10 minutes at room temperature and resuspended in 100 mM Tris-Cl pH 8.0 containing 5mM EDTA and 20% sucrose (TES buffer) at 80ml per gram wet weight. The cells were shaken at 200rpm at room temperature for 10 minutes. The suspension was then centrifuged again and the pellet

5   resuspended in 5 mM ice-cold MgSO₄ (80 ml per gram wet weight). This was shaken for 30 minutes on ice and subsequently centrifuged at 8000 ×_g_ at 4°C for 10 minutes. The supernatant constituting the periplasmic fraction was further purified using the coil affinity column, as described in Example 8, below.

10   ### EXAMPLE 7

Expression of Coil Constructs in Yeast

The E-coil gene described above was used as a template for the polymerase chain reaction (PCR: Saiki, _et al.,_ 1988; Mullis, 1987; Mullis, _et al.,_ 1987) to generate an E-coil gene with a 5' _EcoRI_ site and 3' _NotI_ site. Synthetic Ecopri (SEQ ID NO:12) and Notpri

15   (SEQ ID NO:13) oligonucleotides were used as primers at an annealing temperature of 50°C for 30 cycles.

The PCR amplification product was cloned into plasmid pIC9 (Invitrogen, San Diego, CA), which contained the alpha mating factor leader sequence for secretion into the medium, generating _Pichia_ E-coil vector p9CE. Successfully-ligated plasmids were

20   transformed into _E. coli_ cloning strain TOP10 F' and plated on LB/Carbenicillin (100 μg/ml) plates. Plasmid DNA was prepared from selected colonies and checked for the correct size of insert by digestion with restriction enzymes and confirmed by DNA sequence analysis.

Verified yeast plasmids were transformed into _Pichia pastoris_ strain GS115 as

25   described (Cregg, _et al.,_ 1985). GS115 was cultured in YPD (1% peptone, 2% yeast extract, 2% dextrose) media (Romanos, _et al.,_ 1991) at 30°C with shaking to an A₆₀₀ of 0.25 and harvested by centrifugation. The cell pellet underwent a series of washes with dH₂O and 1M aqueous sorbitol, followed by 1M aqueous sorbitol containing 25mM EDTA and 100 mM dithiothreitol (SED buffer) and again with 1M sorbitol. The final pellet was

30   resuspended in 1M aqueous sorbitol containing 10 mM sodium citrate buffer, pH 5.8, and 1mM EDTA (SCE buffer) and treated with zymolase at a final concentration of 0.3mg/ml.

Spheroplasting was carried out at 30°C and monitored by treating aliquots taken at various time points with 5% SDS and checking their absorbance at 800nm. At 70% spheroplasting, the cells were harvested by centrifugation and washed with 1M aqueous

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sorbitol, followed with a wash in 1M aqueous sorbitol containing 10 mM Tris-HCl, pH 7.5 and 10 mM CaCl₂ (CaS buffer). The spheroplasts were resuspended in CaS and used immediately for DNA transformation.

In preparation for transformation, 10 μg of plasmid p9CE DNA was digested with
5   restriction enzyme *Bg/*II to generate two linear fragments, confirmed by electrophoresis on a 0.7% agarose gel. Newly-prepared spheroplasts were incubated with digested DNA for 10 minutes at room temperature. A fresh solution of 20% PEG with 10 mM CaCl₂ and 10 mM Tris-HCl, pH 7.5 (PEG/CaT) was added and incubation continued for another 10 minutes.

10 Cells were harvested at 750 ×g at room temperature and the supernatant aspirated off. The pellet was resuspended in SOS media (1 M sorbitol, 0.3X YPD media, 10 mM CaCl₂) and the cells were allowed to recover for 20 min before addition of 1M aqueous sorbitol and plating on selective RDB plates (1 M Sorbitol, 1% dextrose, 1.34% yeast nitrogen base, 4 × 10-5% biotin and 0.005% amino acids without histidine). Recombined
15 clones were identified by growth in the absence of histidine, and displacement of the chromosomal AOX1 gene was distinguished by growth on selective minimal methanol media (1.34% yeast nitrogen base, 4 × 10-5% biotin, 0.5% methanol). Clones that had undergone successful integration of the linerarized plasmid fragment were identified by PCR screening (Clare, *et al.*, 1991) using vector-specific primers (SEQ ID NO:14, SEQ ID
20 NO:15).

Recombinant yeast clones exhibiting a methanol sensitive phenotype (MetS) were cultured in BMGY media (peptone, yeast extract, yeast nitrogen base, biotin, glycerol) to an A₆₀₀ of 1.0. The carbon and energy source was subsequently changed to methanol (BMMY media) to allow for induction using the AOX1 promoter (Ellis, *et al.*, 1985). After 72
25 hours of induction, the culture supernatant was harvested by centrifugation at 4000 ×g and filtered through a 0.2 μm filter in preparation for coil affinity purification, described in Example 8, below.

EXAMPLE 8
30   Purification of Proteins on Selective
Dimerization Affinity Matrix Affinity Column

This section describes preparation and use of an affinity matrix designed for purification of polypeptides containing a coil peptide. The affinity purification procedure is based on selective dimerization of a heterodimer-subunit peptide immobilized on a solid
35 phase to a proteins or peptides containing a complementary heterodimer subunit.

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Particularly. polypeptides selected f r purification are fusion peptides designed and synthesized to contain a heterodimer-subunit peptide complementary to the immobilized peptide.

Generally. to prepare a selective dimerization affinity matrix. one of a pair of
5   complementary peptide subunits is synthesized as a linear peptide according to methods known in the art. purified. then conjugated to a solid matrix through one of the residues present in the peptide. Preferably. a cysteine residue will be available for conjugation of the peptide.

The sections that follow describe preparation of an affinity matrix having conjugated
10   thereto a heterodimer-subunit peptide. Also detailed below are conditions used for binding the complementary heterodimer-subunit peptide to the affinity matrix of and elution of the complementary subunit from the column.

A.   Preparation of Selective Dimerization Affinity Matrix
15   1.   Preparation of Peptides. Complementary peptides were designed as described above to form a stable α-helical coiled-coil heterodimer. For conjugation to the solid phase. one of the peptide subunits was synthesized to include a spacing group, shown as the C-terminal 5 amino acids in SEQ ID NO:27. and to have a C-terminal cysteine-amide for convenience of chemical coupling to the matrix.
20   The following pair of complementary peptides was designed and prepared as follows:
SEQ ID NO:26 (Peptide 0993):
Ac-EVSALEKEVSALEKEVSALEKEVSALEKEVSALEK-Amide
SEQ ID NO:27 (Peptide 0994):
25   Ac-KVSALKEKVSALKEKVSALKEKVSALKEKVSALKEGGGnLC-Amide
       gabcdefgabcdefgabcdefgabcdefgabcdef

The peptides were synthesized on a solid phase peptide synthesizer (430A Applied Biosystems Inc.; Foster City, CA) using standard t-BOC chemistry. Peptide was cleaved
30   from the synthesis resin by treatment with hydrogen fluoride. Purification of each peptide was carried out using reversed phase high performance liquid chromatography (RP-HPLC).

2.   Coupling of Peptide to Affinity Matrix. Control pore aminopropyl glass resin (220 mg (22 μmol); Sigma, G-4643, average pore: 500 Angstrom, 200-400

35

mesh. amine content 110umol/g glass) was washed twice with 5.0 ml each of DMF. DCM and DMF. The resin was neutralized with 5.0 ml of a DIEA/DCM solution (5% v/v) for 5.0 minutes and then drained.

The resin was next stirred for 25 minutes in a solution of bromoacetic acid

5 dissolved in DCC/DCM solution, prepared by dissolving 138 mg (1.0 mmol) of Bromoacetic acid (Aldrich Fine Chemicals Catalog #25,935-7) in 2.0 ml of a 0.5 M DCC/DCM solution. stirring for fifteen minutes and filtering to remove insoluble DCU. The resin was again drained and washed three times each with 5.0 ml DCM. DMF. MeOH. DCM. The resulting activated glass resin was then dried.

10 Peptide 0994 (SEQ ID NO:27; 26 mg, 6.15 μmol) was dissolved in 3.0 ml of a 100 mM NH$_4$HCO$_3$ solution. pH 8.0. This solution was added to the activated glass resin beads. The peptide resin solution was stirred for 1.5 hours. after which the resin was drained and washed with 5.0 ml H$_2$O.

Remaining unreacted bromoacetyl groups were capped by adding 5.0 ml of a 1%

15 v/v solution of β-mercaptoethanol in 100 mM NH$_4$HCO$_3$ and the resin mixture stirred for thirty minutes. The resin was then drained and washed 5x with H$_2$0. DMF, and methanol. then air dried for storage. Coupling was verified by qualitative ninhydrin test.

B.   Preparation of Affinity Column

20 An RP-HPLC guard column (5 mmx20 mm) was dry-packed with the prepared dimerization chromatography resin. The column was then washed for 10 minutes at a flow rate of 0.2 ml/min with the following solutions. H$_2$O. 50% acetonitrile-H$_2$O. 5.0 M GndHCl in 50 mM potassium phosphate pH 7.8. and H$_2$O. This extensive washing was carried out in order to remove from the column any non-specific. noncovalently attached

25 peptide.

C.   Chromatography of Heterodimer-Subunit on Selective Dimerization Affinity Matrix

An affinity column prepared as detailed in Part B. above. was equilibrated in 100%

30 H$_2$0 at a flow rate of 0.2 ml/min. A 50 μl sample of peptide 0993 (SEQ ID NO:26; 1 mg/ml in 10 mM phosphate buffer. pH 6.5) was injected and eluate fractions were collected for analysis by RP-HPLC. RP-HPLC was carried out on a C-8 Zorbax analytical column equilibrated with 0.05% TFA in H$_2$0, and eluted with a gradient of 2%/min solution B (0.05% TFA acetonitrile) at a flow rate of 1ml/min and detection at 210 nm.

35 Following injection of sample onto the affinity column. the column was washed with a

solution of 60% acetonitrile/H₂O for 5.0 min. and the eluate during the wash step was

collected for analysis on RP-HPLC. The peptide was subsequently eluted from the affinity

column with a solution of 5.0 M GndHCl in 50mM potassium phosphate pH 7.8. followed

by several volumes of H₂O.

5   Figure 7A shows a chromatogram of peptide 0993 eluting from an RP-HPLC

column at about 25 minutes.

        Figure 7B shows a chromatogram of the injection flow through fraction analyzed by

RP-HPLC under the conditions described above. after injecting peptide 0993 onto the

selective dimerization affinity column described in Part C, above. Absence of a peak at 25

10   min shows that substantially all loaded peptide bound to the column.

        Figure 8 shows a chromatogram of the 60% acetonitrile wash fraction of the affinity

column. The presence of only a minor peptide peak at the peptide 0993 position indicates

that the peptide was not eluted by the washing procedure.

        Figure 9 shows a chromatogram of the GndHCl elution fraction. The major peak

15   eluting at approximately 25 minutes is peptide 0993, indicating that 0.5 M GndHCl is

effective to elute this fraction.

      D.   Conditions for Removal of non-specific Peptides from Selective
                Dimerization Affinity Column

20   Studies were carried out in support of the present invention to determine conditions

for removing non-specific binding of charged peptides from the affinity column. The

following peptides were used in a test mixture:

Peptide Test Mixture:

Peptide	Sequence	Net charge at pH 6.0
SEQ ID NO:28 (Actin 1 -28)	Ac-DEDETTALVADNGSGLUKAGFAGADAPR-Amide	-4
SEQ ID NO:29 (Anion Exchange Standard)	Ac-EYAAEAAEGLE-Amide	-4
SEQ ID NO:30 (TnI 104-115)	Ac -GKFKRPPLRRVR-Amide	+6

        To test for non-specific binding. the affinity column was equilibrated in 10 mM

phosphate pH 6.5 at a flow rate of 0.2ml/min.

        A 100 μl sample of the peptide test mixture (1 mg/ml Actin 1-28. 1 mg/ml Anion

exchange std.. 2 mg/ml TnI 104-115 in 10 mM phosphate buffer, pH 6.5) was injected and

40   the eluate during injection collected for analysis on RP-HPLC. Figure 10 shows a

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chromatogram of the peptide mixture, run on the RP-HPLC column prior to loading on the affinity column. The conditions for running this column were identical to those detailed in Part D, above. Indicated in the figure are 3 major peaks representing the three above-referenced peptides in the mixture.

5   The affinity column was then washed with a solution of 0.2 M KCl/ 10 mM phosphate for 5.0 min and the eluate during the wash step collected for analysis on RP-HPLC. Figure 11 shows a chromatogram of the eluate fraction. The single major peak eluting at about 12 minutes indicates that TnI 104-115 peptide (SEQ ID NO:28) having a net positive (+6) charge was not retained by the column, while both the negatively charged

10   proteins were retained.

·   The affinity column was then washed with a solution of 0.5 M KCl/ 10 mM phosphate for 5.0 min and the eluate during the wash step collected for analysis on RP-HPLC. Figure 12 shows no detectable peptide peaks eluting at positions corresponding to any of the components of the peptide mixture.

15   The affinity column was then washed with a solution of 1.0 M KCl/ 10 mM phosphate for 5.0 min and the eluate during the wash step collected for analysis on RP-HPLC. The chromatogram depicted in Figure 13 shows that both the anion exchange standard peptide (SEQ ID NO:29) and the actin 1-28 peptide (SEQ ID NO:30) were eluted by this step.

20   Subsequent washes were carried out, using (a) 1.0 M KCl, and (b) a solution of 5.0 M GndHCl in 50mM K_2PO_4 pH 7.8. These fractions were collected for RP-HPLC analysis as described above. Figures 14 and 15 show that no additional test peptides were eluted from the column under these conditions.

25   E.   Purification of Recombinant PAK pilin/E-coil peptide

This section describes purification of a recombinant PAK pilin/E-coil fusion protein. The fusion protein was produced as detailed in Example 9.

1.   Preparation of Peptide Affinity Matrix. One gram (110 μmol) of

30   aminopropyl control pore glass resin (Sigma , Cat# G-4643) was washed 2x with 15.0 ml of DMF, DCM and DMF. The resin was neutralized with 15.0 ml of a 5% v/v DIEA/DCM solution for 5.0 minutes and then drained. Two ml of a 0.5 M dicyclohexylcarbodiimide/DCM solution were added to 138 mg (1.0 mmol) of a 0.5 M dicyclohexylcarbodiimide/DCM solution, and the mixture was stirred for fifteen minutes.

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The mixture was then filtered. and the filtrate added to the prepared glass resin beads. The final volume of the mixture was brought up to 20 ml with dichloromethane. The mixture was stirred for 25 minutes and then drained. The resin was washed 3x with 15.0 ml DCM. methanol, and DCM, then dried. A qualitative ninhydrin test was

5   performed to verify coupling. One hundred milligrams (23.7 μmol) of peptide 0994 was dissolved in 30.0 ml of a 100 mM NH$_4$HCO$_3$, pH 8.0 and then added to the glass resin beads. The peptide resin solution was stirred for 30 minutes. after which the resin was drained and washed with 5.0 ml H$_2$O. Remaining unreacted bromoacetyl groups were capped by adding 20.0 ml of a 1% v/v solution of β-mercaptoethanol in 100 mM

10   NH$_4$HCO$_3$, pH 8.0 to the resin, and the mixture was stirred for fifteen minutes. The resin was then drained and washed 5x with H$_2$O. 0.1% TFA H$_2$O. Ethanol, Acetone and then dried for storage. A small amount of resin was removed for amino acid analysis and theoretical capacity determination.

15   2.   Preparation of Affinity Column. Two columns were prepared for affinity purification. i) a stainless steel RP-HPLC guard column (5 mm × 20 mm), and ii) a stainless steel RP-HPLC column (10 mm × 200 mm). Each column was dry-packed to the top with the prepared dimerization chromatography resin. After packing. each column was washed at a flow rate of 0.5 ml/min with the following solutions, H$_2$O. 5.0 M

20   GndHCl in 50 mM potassium phosphate pH 7.0. 50% acetonitrile/H$_2$O with 0.1% TFA. and H$_2$O.

              3.   Purification of Recombinant Peptides. The columns were first conditioned in 10 mM phosphate buffer, pH 6.0. Filtered periplasmic fractions or whole

25   cell extracts were loaded at a flow rate of 0.2-0.5 ml/min. After loading, the columns were washed with 5.0 ml (small column) or 20 ml (large column) of 0.5 M KCl in 10 mM phosphate buffer pH 6.0 followed by 80% acetonitrile in 10 mM phosphate buffer pH 6.0 (v/v). Final elution of bound peptides was carried out with a wash of 50% acetonitrile/H$_2$O with 0.1%TFA (v/v/v).

30   Presence of recombinant protein in eluted fractions was assessed by RP-HPLC (Zorbax C-8 analytical column (10 mm × 250 mm), at a flow rate of 1.0 ml/min with a linear AB gradient of 1.0% B/min from 0% to 60% B, where solvent A was 0.05% trifluoroacetic acid (TFA) in water and solvent B was 0.05% TFA in acetonitrile. The total run time was 60.0 min.

Figure 17 shows RP-HPLC chromatograms of load samples and fractions eluted
from the affinity column. when the load fraction was a crude periplasmic extract containing
recombinantly expressed E-coil peptide. HPLC runs are indicated as follows: a. pre-
elution: b. crude periplasmic extract, c. break-through of periplasmic extract; d. 0.5 M KCl

5   wash: e. 80% acetonitrile wash: f. first elution wash; g. second elution wash: h. first
elution wash after re-passage of break-through. followed by washes.

Figure 18 shows RP-HPLC chromatograms of purification of recombinantly
expressed PAK-pili-E-coil peptide from crude periplasmic extract. All HPLC runs were
carried out as described above. HPLC runs are indicated as follows: a. pre-elution: b.

10   crude periplasmic extract. c. break-through of periplasmic extract: d. 0.5 M KCl wash: e.
80% acetonitrile wash: f. first elution wash; g. second elution wash.

EXAMPLE 9

PAK Pili Antigen/E-Coil Fusion

15   Two complementary oligonucleotides (oligonucleotides SEQ ID NO:16 and SEQ ID
NO:17) encoding the 17 amino acid pilus epithelial binding domain (SEQ ID NO:5) of the
PAK pili (128-144: Lee. *et al.*, 1994) were synthesized. Equal molar ratios of the
oligonucleotides were dissolved in sterile dH_20. mixed together. heated to 80°C for 5 min
and allowed to anneal by slow cooling to room temperature. The annealed oligonucleotides

20   were electrophoresed on a 4% agarose gel and a 54 bp fragment was excised and eluted
from the gel slice by a modified freeze-squeeze method (Sambrook. *et al.*. 1989). Briefly.
the excised band was placed in a spin filter column (BioRad. Richmond. CA) and placed on
liquid nitrogen for 5 minutes. The filter was then placed into an eppendorf tube and spun at
top speed (~ 14.000 rpm) in a microfuge.

25   Following ethanol precipitation. the eluate was treated with T4 polynucleotide
kinase at 37°C for 30 minutes and the enzyme inactivated at 65°C for 15 min. The
phosphorylated fragment was then cloned into shrimp alkaline phosphatase-treated. *EcoRI*-
digested pRLDE and transformed into *E. coli* strain JM83 to produce pRLDE-PAK clones
on LB/Carbenicillin plates.

30   Orientation and sequence of the insert was verified by restriction analysis and
sequencing of minipreped DNA plasmid. Expression and purification of the PAK/E-coil
fusion protein was carried out as previously described for the E-coil protein.

40

EXAMPLE 10

Green Fluorescence Protein/E-Coil Fusion

A Green Fluorescent Protein (GFP) cDNA clone (Prasher, et al., 1992) was purchased from ClonTech (Palo Alto, CA). GFP-specific PCR primers (oligonucleotides

5   SEQ ID NO:18 and SEQ ID NO:19) were constructed with flanking EcoRI restriction sites and PCR of the GFP gene was carried out under standard conditions (Saiki, et al., 1988). The PCR product was run on a 1% agarose gel and the 700bp amplification product was excised and eluted from the gel as described above.

Plasmid pRLDE-GFP was constructed by digesting the eluted DNA fragment with

10 EcoRI and ligating the DNA into shrimp alkaline phosphotase-treated, EcoRI-digested pRLD-E. pRLDE-GFP was then transformed into E. coli strain TOP 10 F'. Orientation of the gene was determined by direct detection of fluorescent colonies on LB/CA/IPTG transformation plates (100 μg/μl carbenicillin, 0.8mM IPTG). The sequence of the gene was confirmed by restriction analysis and sequencing. Expression and purification of the

15 protein was carried out as previously described.

EXAMPLE 11

Western and Ligand Blot Analyses of
PAK/E-coil Fusion

20   Crude periplasmic prep from E. coli JM 83 cells containing expressed PAK 17 mer peptide tagged with E-coil, and PAK17/E-coil purified by the K-coil affinity column were fractionated by SDS-PAGE at a constant voltage of 200V with a power supply model 1420A (BioRad Laboratories). Figure 16A shows an image of the gel stained with Coomassie blue. The lanes are as follows: lane 1: crude PAK(128-144)/E-coil periplasmic

25 prep; lane 2: PAK (128-144)/E-coil purified by K-coil affinity column; lane 3: rainbow marker (2-45 kda).

The gel was blotted and subjected to Western Blot analysis as described above. Monoclonal antibody PK99H, raised against the Pseudomonas aeruginosa strain K pili (PAK pili), was diluted with TTBS (1:500), and incubated with the blots for one hour at room

30 temperature with gentle shaking. The blots were then washed three times with TTBS, and a goat anti-mouse IgG(H+L)-alkaline phosphatase conjugate (Jackson Laboratories) diluted 1:10,000 with TTBS was incubated with the blots as described above.

The blots were washed 3 times with TTBS followed by a final wash with Tris-buffered saline. Antibody binding was visualized by the addition of alkaline

35 phosphatase substrates (nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl

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phosphate dissolved in 100 mM Tris-HCl pH 9.5. containing 100 mM NaCl and 5 mM MgCl₂). Color development was stopped by rinsing the nitrocellulose strips with deionized water.

Ligand-blots were performed to evaluate the ability of biotin-labelled (bt) K-coil to
5   act as a probe. A gel as shown in Figure 16A was blotted as described above. and biotin-labelled K-coil (1 mg/ml) was diluted 1:500 in PBS-ED blocking buffer and incubated with the blots at room temperature for 1 hr with gentle agitation. The membranes were washed three times. 10 minutes per wash.

Strepavidin-alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratory)
10 was diluted (1:2500; vol/vol) with PBS-ED blocking buffer and incubated with the blots at room temperature for 1 hr with gentle agitation. The blots were washed 3 times with PBS-ED followed by a final wash with substrate buffer (100 mM Tris-HCl pH 9.5. 100 mM NaCl and 5 mM MgCl2). K-coil binding was visualized by the addition of nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in substrate
15 buffer. Color development was stopped by rinsing the nitrocellulose strips with deionized water.

The results of the Western and ligand Blot analyses are shown in Figure 16B. Lanes 2-3 and 8-9 were probed using the ligand blot technique. while lanes 5-6 and 11-12 were probed using the Western blot technique. Lanes 1. 4. 7 and 10 contained size markers
20 (Rainbow marker: 2-45 kDa): lane 2: bt K-coil binding to crude PAK(128-144)/E-coil periplasmic prep: lane 3: bt K-coil binding to PAK(128-144)/E-coil purified by K-coil affinity column: lane 5: monoclonal antibody. PK99H. binding to crude pak (128-144)/E-coil periplasmic prep: lane 6. monoclonal antibody. PK99H. binding to purified PAK (128-144)/E-coil by K-coil affinity column: lane 8. bt K-coil binding to E-coil purified by K-coil
25 affinity column: lane 9. Bt-K-coil binding to PAK pili: lane 11. monoclonal antibody, PK99H. binding to E-coil purified by K-coil affinity column; lane 12. normal mouse IgG binding to PAK pili.

EXAMPLE 12

Isolation of PAK/E-coil Fusion Using
30   K-coil Affinity Column

A.   Isotope (¹⁵N) Labelling

A single colony of *E. coli* strain JM83 harboring the recombinant expression plasmids (pRLDE-PAK) was inoculated in 5 ml LB medium contain 100 μg/ml carbenicillin
35 (Sigma) and grown at 37°C overnight. The overnight culture (2 ml) was sub-inoculated in

42

200 ml of mini-medium (0.6% NaH$_2$PO$_4$. 0.3% K$_2$HPO$_4$. 0.05% NaCl. 4 mM MgSO$_4$. 2 μM FeSO$_4$. 0.1% ^{15}NH$_4$Cl (Cambridge Isotope Laboratories. Woburn. MA) and 1% D-glucose, pH 7.2). The inducer. IPTG. was added to culture to final concentration of 1 mM once OD$_{600}$ reached 0.8 to 1.0. The cultures were grown at 37°C for another 6 hr and 5 applied for further purification.

B.   Preparation of _E. coli_ Periplasmic Prep

The induced gene-expression cultures were harvested by centrifugation (4000 ×g. 10 min). The pellets were resuspended in 100 ml of TES buffer containing 100 mM Tris. 10 10 mM EDTA and 20% sucrose and incubated at room temperature for 10 min with gentle agitation. The cells were collected by centrifuging (7000 ×g. 10 min) and resuspended in 5 mM MgSO$_4$. The suspension was incubated in ice-cool bath for 30 min with agitation. The supernatant which contained the periplasmic proteins was collected by centrifugation (5000 ×g. 10 min) and stored at -20°C or freeze-dried.

15

C.   HPLC-Affinity Column for Purification of PAK/E-coil

The freeze-dried expressed crude periplasmic preps were dissolved in 20 ml of 10 mM phosphate buffer (pH 6). The dissolved sample (5 ml) was loaded on an affinity column conjugated with K-coil peptide after the column was washed and equilibrated with 20 10 mM phosphate buffer (pH 6).

The constant flow rate of HPLC was 0.2 ml/min. The column was washed by injecting 5 ml 0.5 M KCl (pH 6) in phosphate buffer 25 minutes after the sample was loaded. Five ml of 80% acetonitrile in 10 mM phosphate buffer pH 6.5 were injected 25 min after the 0.5 M KCl washing. The PAK/E-coil fusion was eluted with 5 ml of 50% 25 acetonitrile containing 0.1% TFA in ddH$_2$O. The identity of the eluted sample was confirmed using reversed phase chromatography and mass spectrometer analysis.

EXAMPLE 13

Detection Reagent

30   This section describes synthesis and use of a reagent for detecting fusion proteins having a heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a complementary peptide in conformance with the invention.

43

A.   Iodinated Reagent

A heterodimer-subunit peptide is formed as described above. The peptide is then iodinated according to standard procedures known in the art. Preferably, heterodimer-subunit peptide designed for iodination contains a tyrosine residue; however, iodination can also be accomplished by use of, e.g., a Bolton-Hunter Reagent, which preferably attaches to lysine residues. For example, lysines occupying position f of the subunit repeat unit may be so derivatized. Kits containing Bolton-Hunter reagent are commercially available (e.g., ICN, Costa Mesa, CA).

Another iodination method is the "IODOGEN" method. Here, 2 mCi of carrier-free $Na^{125}I$, 75 μl 0.5M phosphate buffer pH 7.4 and 20 μl of 1 $\mu g/\mu l$ peptide are added to a polypropylene test tube coated with 10 μg "IODOGEN". The tube is agitated for 8 minutes, and the solution is chromatographed by HPLC through a 10 × 0.46 cm C-8 reversed phase column with a pore size of 300 Å (Brownlee Labs, Santa Clara, CA). The sample material is eluted with a gradient from 0.1% trifluoroacetic acid to 60% acetonitrile in 0.1% trifluoroacetic acid. The major peak of active radio-iodinated peptide is detected by gamma-detection of fractions and is used as a reporter molecule in the method of the invention.

B.   Enzyme-linked Reagent

An enzyme linked reagent can be made according to methods well known in the art. In one such method, a gene for the desired reporter enzyme, such as alkaline phosphatase or luciferase, is fused to a gene for the heterodimer subunit, and the expressed fusion protein purified by affinity chromatography on a complementary heterodimer-subunit peptide affinity column, according to the methods described above.

In another method, the heterodimer-subunit peptide is chemically linked to the reporter enzyme. For example, horseradish peroxidase (HRP) can be conjugated to a cysteine residue present in the peptide subunit, preferably placed in the f heptad position of the coil peptide, through a disulfide bond. In one method, HRP (6mg/ml in 0.1M sodium phosphate buffer, pH 7) is incubated for 0.5-1 hour at 30°C with 0.3-0.7 mg of cross-linking reagent (N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) or N-succinimidyl 6-maleimidohexanoate (SMH)) dissolved in N,N-dimethylformamide (10% solution). The reacted HRP is isolated by gel filtration of Sephadex G-25 (equilibrated with 0.1M phosphate, pH 6; Pharmacia, Piscataway, NJ). To couple the reactive HRP with a reduced cysteine-containing heterodimer subunit, the two components

are mixed at a 1:1 molar ratio and a final concentration of 0.01-0.15 mM in 0.1 M sodium

phosphate buffer, pH 6 containing 2 mM EDTA, at 4°C for 20 hours or at 30°C for 1

hour. The conjugated product can be purified on "ULTROGEL" AcA 44 (Pharmacia

Biotech, Piscataway, NJ).

5   The HRP-conjugated heterodimer-subunit peptide acts as a reporter molecule when

it is reacted with 4-aminoantipyrine as substrate and increased absorbance at 510 nm is

measured.

While the invention has been described with reference to specific methods and

embodiments, it will be appreciated that various modifications and changes may be made

10   without departing from the invention.

45

SEQUENCE LISTING

(1) GENERAL INFORMATION:

     (i) APPLICANT: PENCE

     (ii) TITLE OF INVENTION: Coiled-Coil Heterodimer Methods and
                 Compositions for the Detection and Purification
                 of Expressed Polypeptides

     (iii) NUMBER OF SEQUENCES: 30

     (iv) CORRESPONDENCE ADDRESS:
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     (v) COMPUTER READABLE FORM:
              (A) MEDIUM TYPE: Floppy disk
              (B) COMPUTER: IBM PC compatible
              (C) OPERATING SYSTEM: PC-DOS/MS-DOS
              (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

     (vi) CURRENT APPLICATION DATA:
              (A) APPLICATION NUMBER: PCT/US96/
              (B) FILING DATE: 04-OCT-1996
              (C) CLASSIFICATION:

     (vii) PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER: US 08/540,397
              (B) FILING DATE: 06-OCT-1995

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 105 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: double
       (D) TOPOLOGY: both

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (C) INDIVIDUAL ISOLATE: E-coil sequence

    (ix) FEATURE:
       (A) NAME/KEY: CDS
       (B) LOCATION: 1..105

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAG GTA TCC GCT TTA GAG AAA GAA GTT TCT GCT CTC GAA AAA GAG GTC        48
Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val
 1               5                  10                  15

AGT GCT CTG GAA AAA GAG GTG TCA GCC TTG GAA AAG GAA GTA TCA GCA        96
Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala
            20                  25                  30

CTT GAG AAG                                                           105
Leu Glu Lys
        35
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 35 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val
 1               5                  10                  15

Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala
            20                  25                  30

Leu Glu Lys
        35
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 105 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: double
       (D) TOPOLOGY: both

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

47

```
        (iv) ANTI-SENSE: NO

        (vi) ORIGINAL SOURCE:
             (C) INDIVIDUAL ISOLATE: K-coil sequence

        (ix) FEATURE:
             (A) NAME/KEY: CDS
             (B) LOCATION: 1..105


        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AAG GTA TCC GCT TTA AAA GAG AAA GTT TCT GCT CTG AAA GAA AAG GTC          48
Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val
1               5                   10                  15

AGT GCT CTG AAG GAG AAG GTG TCA GCC TTG AAG GAA AAG GTT TCA GCA          96
Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala
            20                  25                  30

CTT AAA GAG                                                             105
Leu Lys Glu
        35


(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val
1               5                   10                  15

Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala
            20                  25                  30

Leu Lys Glu
        35


(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 17 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (vi) ORIGINAL SOURCE:
             (C) INDIVIDUAL ISOLATE: PAK peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Cys Thr Ser Asp Gln Asp Glu Gln Phe Ile Pro Lys Gly Cys Ser
1               5                   10                  15

Lys
```

48

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 49 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (C) INDIVIDUAL ISOLATE: Oligonucleotide #1

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAATTCGGA GGAGGTGGAG GTGGTGGTGG CGAGGTATCC GCTTTAGAG   49

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 65 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (C) INDIVIDUAL ISOLATE: Oligonucleotide #2

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTCGTATCAA GAGCACTGAC CTCTTTTTCG AGAGCAGAAA CTTCTTTCTC TAAAGCGGAT   60

ACCTC   65

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 66 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
       (C) INDIVIDUAL ISOLATE: Oligonucleotide #3

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCGTATCAA GTGCTCTGGA AAAAGAGGTG TCAGCCTTGG AAAAGGAAGT ATCAGCACTT   60

GAGAAG   66

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 59 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #4

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGGGATCCTT ATTAGTGATG GTGGTGATGT CCTCCACCGC CCTTCTCAAG TGCTGATAC   59

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 41 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #5

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTCGAGGGTA CCGAATTCCT GCAGAGATCT GCGGCCGCAC A   41

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #6

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTGTGCG GCCGCAGATC TCTGCAGGAA TTCGGTACCC TCGAG   45

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #7

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGAATTCGGA GGAGGTGG   18

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #8

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTCCTTTT GCGGCCGCTT ATTAGTGATG GTGGTG   36

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #9

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACTGGTTCC AATTGACAAG C   21

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #10

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCAAATGGCA TTCTGACATC C   21

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #11

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTCAAGTG TACTTCTGAC CAAGACGAGC AATTCATCCC TAAGGGTTGT TCCA   54

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Oligonucleotide #12

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AATTTGGAAC AACCCTTAGG GATGAATTGC TCGTCTTGGT CAGAAGTACA CTTG   54

52

(2) INFORMATION FOR SEQ ID NO:18:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 31 base pairs
            (B) TYPE: nucleic acid
 5   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA

10   (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
15   (C) INDIVIDUAL ISOLATE: Oligonucleotide #13

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

      GGAATTCATG AGTAAAGGAG AAGAACTTTT C   31
20
(2) INFORMATION FOR SEQ ID NO:19:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
25   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA
30
     (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

35   (vi) ORIGINAL SOURCE:
            (C) INDIVIDUAL ISOLATE: Oligonucleotide #14

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

40   GGAATTCTTT GTATAGTTCA TCCATGCCAT   30

(2) INFORMATION FOR SEQ ID NO:20:

      (i) SEQUENCE CHARACTERISTICS:
45   (A) LENGTH: 15 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: unknown
            (D) TOPOLOGY: unknown

50   (ii) MOLECULE TYPE: DNA

     (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO
55
      (vi) ORIGINAL SOURCE:
            (C) INDIVIDUAL ISOLATE: DNA encoding enterotoxin cleavage site

      (ix) FEATURE:
60   (A) NAME/KEY: CDS
            (B) LOCATION: 1..15

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

65   GAC GAT GAC GAT AAG   15
     Asp Asp Asp Asp Lys
       1   5

(2) INFORMATION FOR SEQ ID NO:21:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 5 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (vi) ORIGINAL SOURCE:
          (C) INDIVIDUAL ISOLATE: Enterotoxin cleavage site

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Asp Asp Asp Asp Lys
  1   5

(2) INFORMATION FOR SEQ ID NO:22:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 12 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: unknown
          (D) TOPOLOGY: unknown

      (ii) MOLECULE TYPE: DNA

      (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (vi) ORIGINAL SOURCE:
          (C) INDIVIDUAL ISOLATE: DNA encoding factor Xa cleavage site

      (ix) FEATURE:
          (A) NAME/KEY: CDS
          (B) LOCATION: 1..12

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATC GAA GGT CGT   12
Ile Glu Gly Arg
  1

(2) INFORMATION FOR SEQ ID NO:23:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 4 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (vi) ORIGINAL SOURCE:
          (C) INDIVIDUAL ISOLATE: Factor Xa cleavage site

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Ile Glu Gly Arg
  1

54

(2) INFORMATION FOR SEQ ID NO:24:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 18 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: unknown
  (D) TOPOLOGY: unknown

 (ii) MOLECULE TYPE: DNA

 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO

 (vi) ORIGINAL SOURCE:
  (C) INDIVIDUAL ISOLATE: DNA encoding thrombin cleavage site

 (ix) FEATURE:
  (A) NAME/KEY: CDS
  (B) LOCATION: 1..18

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CTG GTT CCG CGT GGA TCC   18
Leu Val Pro Arg Gly Ser
 1   5

(2) INFORMATION FOR SEQ ID NO:25:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 6 amino acids
  (B) TYPE: amino acid
  (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: protein

 (vi) ORIGINAL SOURCE:
  (C) INDIVIDUAL ISOLATE: Thrombin cleavage site

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Leu Val Pro Arg Gly Ser
 1   5

(2) INFORMATION FOR SEQ ID NO:26:

 (i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 35 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: unknown

 (ii) MOLECULE TYPE: peptide

 (iii) HYPOTHETICAL: NO

 (iv) ANTI-SENSE: NO

 (vi) ORIGINAL SOURCE:
  (C) INDIVIDUAL ISOLATE: 0993 peptide

 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

 Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val
  1   5   10   15

55

```
          Ser Ala Leu Glu Lys Glu Val Ser Ala Leu Glu Lys Glu Val Ser Ala
                   20                      25                   30

          Leu Glu Lys
                   35
```

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 40 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: 0994 peptide

    (ix) FEATURE:
        (A) NAME/KEY: Modified-site
        (B) LOCATION: 39..39
        (D) OTHER INFORMATION: /label= nLeu
                /note= "where "Leu" is norleucine"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
          Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val
          1               5                   10                  15

          Ser Ala Leu Lys Glu Lys Val Ser Ala Leu Lys Glu Lys Val Ser Ala
                   20                      25                   30

          Leu Lys Glu Gly Gly Gly Leu Cys
                   35                  40
```

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Actin 1-28 peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

```
          Asp Glu Asp Glu Thr Thr Ala Leu Val Ala Asp Asn Gly Ser Gly Leu
          1               5                   10                  15

          Val Lys Ala Gly Phe Ala Gly Ala Asp Ala Pro Arg
                   20                      25
```

(2) INFORMATION FOR SEQ ID NO:29:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: Anion Exchange Standard peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

    Glu Tyr Ala Ala Glu Ala Ala Glu Gly Leu Glu
    1   5   10

(2) INFORMATION FOR SEQ ID NO:30:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: unknown

    (ii) MOLECULE TYPE: peptide

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (C) INDIVIDUAL ISOLATE: TnI 104-115 peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

    Gly Lys Phe Lys Arg Pro Pro Leu Arg Arg Val Arg
    1   5   10

IT IS CLAIMED:

1.   A coding segment. for use in an expression vector suitable for expressing heterologous proteins in a host cell. said segment comprising

(i) a heterologous DNA coding site,

5   (ii) a DNA region encoding a first heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a complementary second heterodimer-subunit peptide. and

(iii) disposed between the coding site and the DNA region. a cleavage sequence, in frame with said DNA region. encoding an amino acid sequence comprising a target for 10 chemical or enzymatic cleavage,

wherein

(A) one of said first and second heterodimer-subunit peptides contains at least two heptad amino acid repeat sequences having the form gabcdef. where positions a and d of each amino acid repeat sequence are selected from the group consisting of leucine,

15 isoleucine and valine. and positions e and g of each amino acid repeat sequence are selected from the group consisting of aspartic acid and glutamic acid,

(B) the other of said first and second heterodimer-subunit peptides contains at least two heptad amino acid repeat sequences having the form g'a'b'c'd'e'f'. where positions a' and d' of each amino acid repeat sequence are selected from the group consisting of

20 leucine. isoleucine and valine. and positions e and g of each amino acid repeat sequence are selected from the group consisting of lysine. arginine and histidine. and

(C) each of the corresponding d/a' and a/d' pairs in complementary heptads of said α-helical coiled-coil heterodimer consists of residues where one of the residues is a valine and the other is selected from the group consisting of leucine and isoleucine.

25

2. The segment of claim 1. wherein said coding site includes a multiple cloning site (MCS) at which a heterologous DNA coding region can be inserted.

3. The segment of claim 1. wherein said coding site includes a heterologous DNA 30 coding region.

4. The segment of claim 1. wherein said DNA region has a sequence selected from the group consisting of the sequences represented by SEQ ID NO:2. SEQ ID NO:4. SEQ ID NO:26 and SEQ ID NO:27.

58

5. The segment of claim 1. wherein said cleavage sequence encodes an
enterokinase cleavage site.

6.   A fusion protein. comprising a selected polypeptide having a functional
5 activity. and attached to the N- or C-terminus of the selected polypeptide. a first
heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a
complementary second heterodimer-subunit peptide.

   wherein

   (A) one of said first and second heterodimer-subunit peptides contains at least two
10 heptad amino acid repeat sequences having the form gabcdef. where positions a and d of
each amino acid repeat sequence are selected from the group consisting of leucine.
isoleucine and valine. and positions e and g of each amino acid repeat sequence are
selected from the group consisting of aspartic acid and glutamic acid.

   (B) the other of said first and second heterodimer-subunit peptides contains at least
15 two heptad amino acid repeat sequences having the form g'a'b'c'd'e'f'. where positions a'
and d' of each amino acid repeat sequence are selected from the group consisting of
leucine. isoleucine and valine. and positions e and g of each amino acid repeat sequence
are selected from the group consisting of lysine. arginine and histidine. and

   (C) each of the corresponding d/a' and a/d' pairs in complementary heptads of
20 said α-helical coiled-coil heterodimer consists of residues where one of the residues is a
valine and the other is selected from the group consisting of leucine and isoleucine.

7. The fusion protein of claim 6. wherein one of the first and second
heterodimer-subunit peptides has a sequence selected from the group consisting of SEQ ID
25 NO:2 and SEQ ID NO:26. and the other peptide has a sequence selected from the group
consisting of SEQ ID NO:4 and SEQ ID NO:27.

8.   A reagent for detecting the presence of an expressed polypeptide,
comprising
30   a second heterodimer-subunit peptide capable of forming an α-helical coiled-coil
heterodimer with a complementary first heterodimer-subunit peptide, where the first
heterodimer-subunit peptide is expressed in tandem with said polypeptide and
       a detectable reporter attached to said second heterodimer-subunit peptide,
       wherein
35   (A) one of said first and second heterodimer-subunit peptides contains at least two

heptad amino acid repeat sequences having the form gabcdef, where positions a and d of
each amino acid repeat sequence are selected from the group consisting of leucine,
isoleucine and valine, and positions e and g of each amino acid repeat sequence are
selected from the group consisting of aspartic acid and glutamic acid,

5   (B) the other of said first and second heterodimer-subunit peptides contains at least
two heptad amino acid repeat sequences having the form g'a'b'c'd'e'f', where positions a'
and d' of each amino acid repeat sequence are selected from the group consisting of
leucine, isoleucine and valine, and positions e and g of each amino acid repeat sequence
are selected from the group consisting of lysine, arginine and histidine, and

10   (C) each of the corresponding d/a' and a/d' pairs in complementary heptads of
said α-helical coiled-coil heterodimer consists of residues where one of the residues is a
valine and the other is selected from the group consisting of leucine and isoleucine.

           9. A method of detecting expression of a selected polypeptide, comprising
15   expressing a fusion protein of claims 6 or 7,
           contacting the fusion protein with a detection reagent composed of a second
heterodimer-subunit peptide and a detectable reporter attached thereto, where the first
heterodimer-subunit peptide is capable of forming an α-helical coiled-coil heterodimer
with the second heterodimer-subunit peptide under the conditions of said contacting, and
20   detecting the presence of said heterodimer.

           10. The method of claim 9, wherein the detection reagent is composed of a fusion
protein containing Green Fluorescent Protein in tandem with the second heterodimer-
subunit peptide.
25

           11. The method of claim 9, wherein one of the first and second heterodimer-
subunit peptides has a sequence selected from the group consisting of SEQ ID NO:2 and
SEQ ID NO:26, and the other peptide has a sequence selected from the group consisting
of SEQ ID NO:4 and SEQ ID NO:27.
30

           12.   A kit for detecting the expression of a selected polypeptide, comprising
           (I) an expression vector comprising
           a replication segment which permits replication of the vector in a selected host
cell, and
35   an expression cassette which contains, in a 5'-3' direction:

(a) a promoter functional in said host cell and

(b) a coding segment of any of claims 1-5. where the coding segment may be oriented with either its coding site or DNA region adjacent said promoter; and
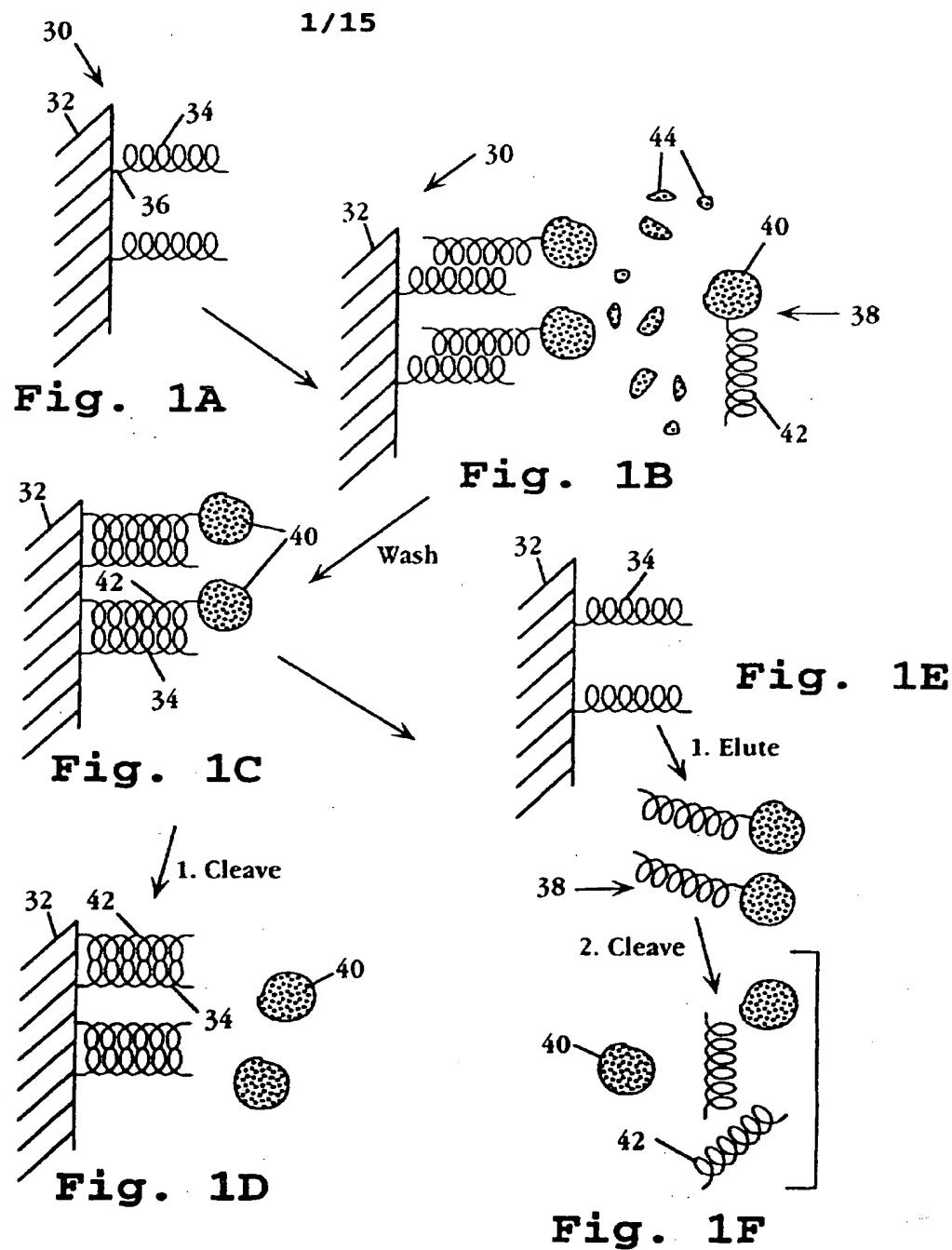
(II) a detection reagent. comprising

5   said second heterodimer-subunit peptide. where the first heterodimer-subunit peptide is expressed in tandem with said polypeptide. and

a detectable reporter attached to said second heterodimer-subunit peptide.

13.   An affinity matrix composition for the purification of a selected
10 polypeptide. comprising

a solid support. and

attached to said support. a second heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with a first heterodimer subunit.

where the selected polypeptide is part of a fusion protein of claims 6 or 7.
15

14.   The affinity matrix composition of claim 13. wherein the solid support is a glass resin.

15.   A method of purifying a selected expressed polypeptide. comprising
20   (i) expressing. in a suitable host-cell expression system. a fusion protein containing said selected polypeptide in tandem with a first heterodimer subunit;

(ii) obtaining from said host cell expression system a suspension containing said fusion protein;

(iii) passing said suspension over an affinity matrix composition containing
25   (a) a solid support. and

(b) attached to said support. a second heterodimer-subunit peptide capable of forming an α-helical coiled-coil heterodimer with said first heterodimer subunit. under conditions that promote immobilization of said fusion protein on said matrix composition by way of formation of an α-helical coiled-coil heterodimer composed of said first and
30 second heterodimer subunits;

(v) washing said affinity matrix while said fusion protein remains bound to said affinity matrix via said α-helical coiled-coil heterodimer. said washing including. in either order.

(a) a salt wash step comprising washing said affinity matrix with a solution having
35 a pH of between about 5.5 and about 8.0 and containing between about 0.1 M and about 1

M salt. and

(b) an organic wash step comprising washing said affinity matrix with a solution
having a pH of between about 5.5 and 8.0 and containing between about 50% and about
100% of an organic solvent; and

5   (vi) releasing the selected polypeptide from said matrix.

16.   The method of claim 15, wherein the expression system is an *E. coli* or
Yeast expression system.

10   17.   The method of claim 15, wherein said releasing includes eluting said
fusion protein with a solution having a pH of less than about 3.0 and containing between
about 20% and about 80% of an organic solvent.

18.   The method of claim 15, wherein said releasing includes eluting said
15 fusion protein with a solution having a pH of more than about 10.0 and containing
between about 20% and about 80% of an organic solvent.

19.   The method of claim 15, wherein said organic solvent is selected from the
group consisting of methanol, ethanol, isopropanol, tetrahydrofuran, trifluoroethanol, and
20 acetonitrile.

20.   The method of claim 19, wherein said organic solvent is acetonitrile.

21.   The method of claim 15, wherein said salt is selected from the group
25 consisting of sodium chloride, potassium chloride, sodium acetate, ammonium chloride,
ammonium acetate, sodium perchlorate, potassium perchlorate, sodium phosphate, and
potassium phosphate.

22.   The method of claim 21, wherein said salt is NaCl.
30

23.   The method of claim 15, wherein one of the first and second heterodimer
subunit peptides has a sequence selected from the group consisting of SEQ ID NO:2 and
SEQ ID NO:26, and the other peptide has a sequence selected from the group consisting
of SEQ ID NO:4 and SEQ ID NO:27.
35

62

24.   A kit for purifying a selected polypeptide. comprising

(I) an expression vector comprising

a replication segment which permits replication of the vector in a selected host

cell. and

5   an expression cassette which contains. in a 5'-3' direction:

(a) a promoter functional in said host cell and

(b) a coding segment of any of claims 1-5. where the coding segment may be

oriented with either its coding site or DNA region adjacent said promoter: and

(II) affinity matrix composition. containing

10   a solid support. and

attached to said support. a second heterodimer-subunit peptide capable of forming

an α-helical coiled-coil heterodimer with said first heterodimer-subunit peptide.

where the selected polypeptide is part of a fusion protein which also contains such

first heterodimer subunit.

15

25.   The kit of claim 24. further including host cells suitable for use with said

expression vector.

Fig. 1A

Fig. 1B

Fig. 1C

Wash

Fig. 1E

Fig. 1D

1. Cleave

1. Elute

2. Cleave

Fig. 1F

Fig. 2

Homodimers

Heterodimers

Stabilized

Fig. 3A

Destabilized

Fig. 3B

Destabilized

Fig. 3C

Stabilized

Fig. 3D

Destabilized

Fig. 3E

Fig. 4A   Fig. 4B   Fig. 4C

OLIGO #1   OLIGO #3
OLIGO #2   OLIGO #4

Fig. 5A   **Fig. 5D**

↓ Klenow   ↓ Klenow

EcoRI   Alw21I   Alw21I   BamHI

Fig. 5B   **Fig. 5E**

↓ dig with Alw21I   ↓ dig with Alw21I

EcoRI   Cut   Cut   BamHI
                   Alw21I   Alw21I

Fig. 5C   **Fig. 5F**

↘   Ligase   ↙

EcoRI   Alw21I   BamHI

Fig. 5G

↓ dig with EcoRI and BamHI

Clone into EcoRI/BglII cut RLD vector

Fig. 5H

Fig. 6A

Fig. 6B

Fig. 7A

Fig. 7B

Fig. 8

Fig. 9

Fig. 10

Fig. 11

Fig. 12

Fig. 13

Fig. 14

Fig. 15

Fig. 16A

Fig. 16B

Fig. 17A

Fig. 17B

Fig. 17C

Fig. 17D

Fig. 17E

Fig. 17F

Fig. 17G

Fig. 17H

RETENTION TIME (minutes)
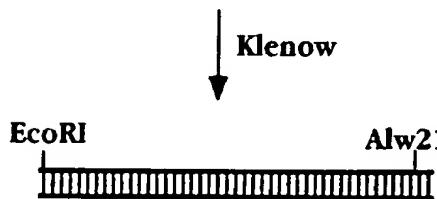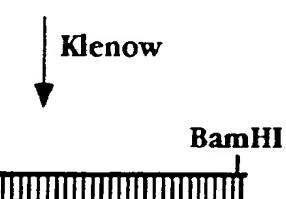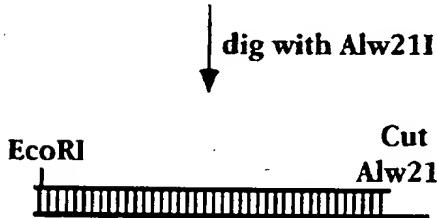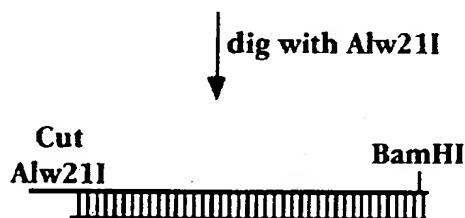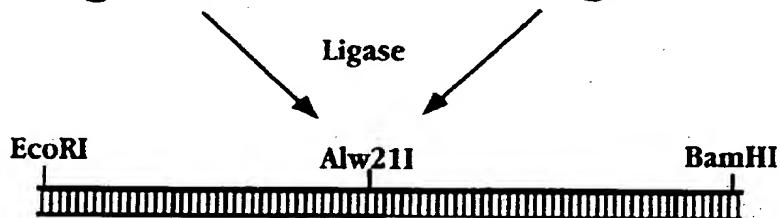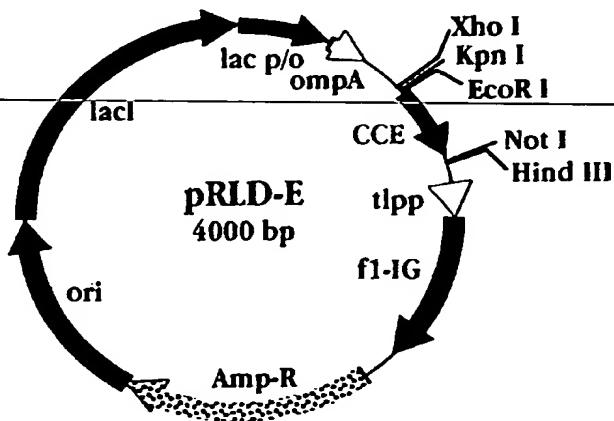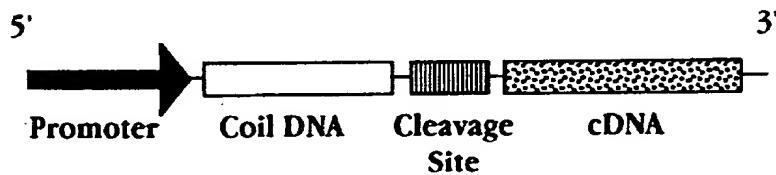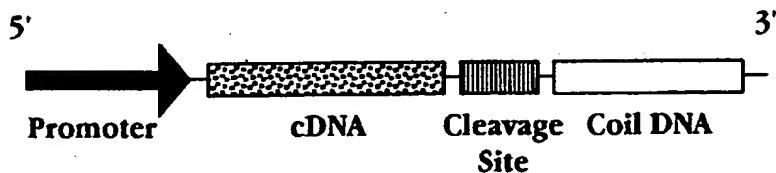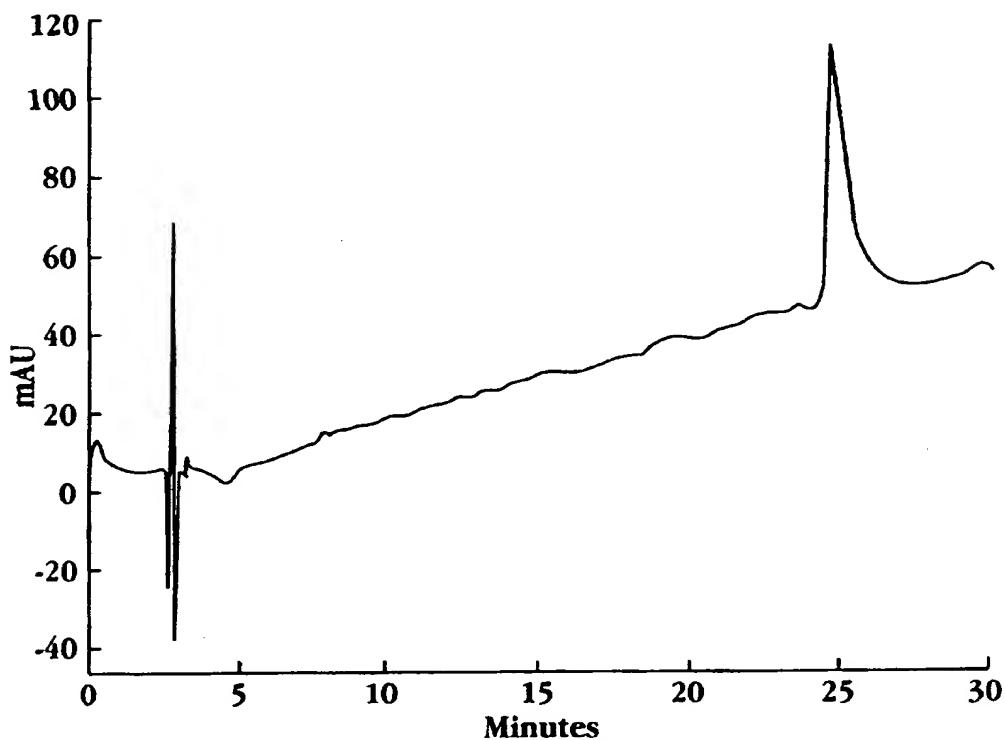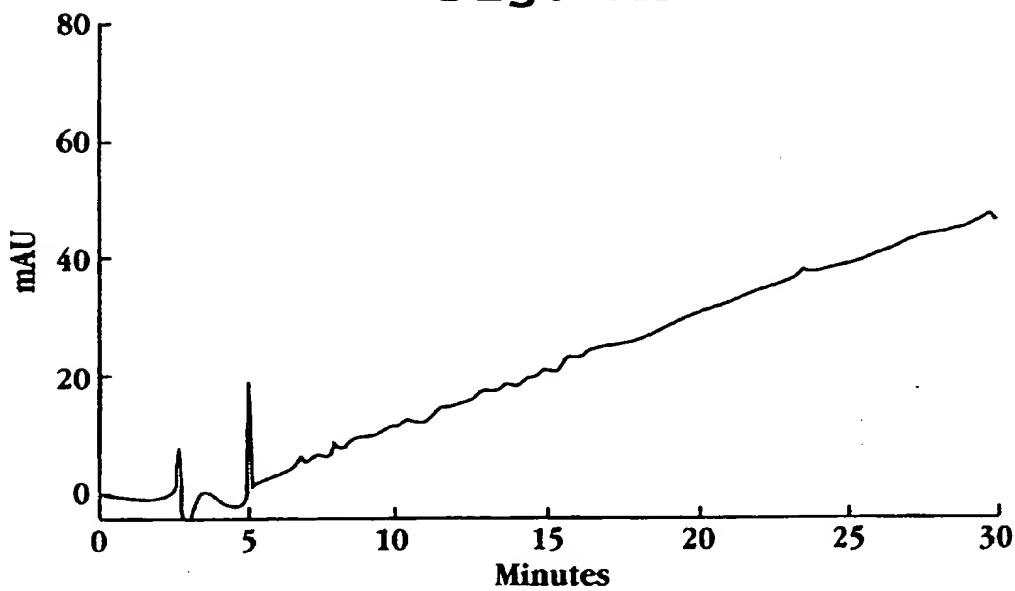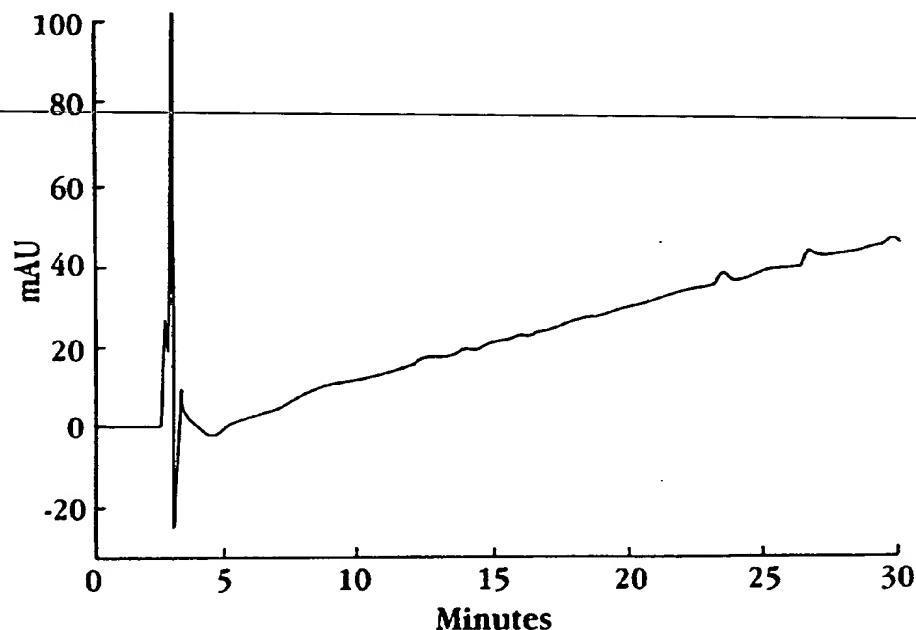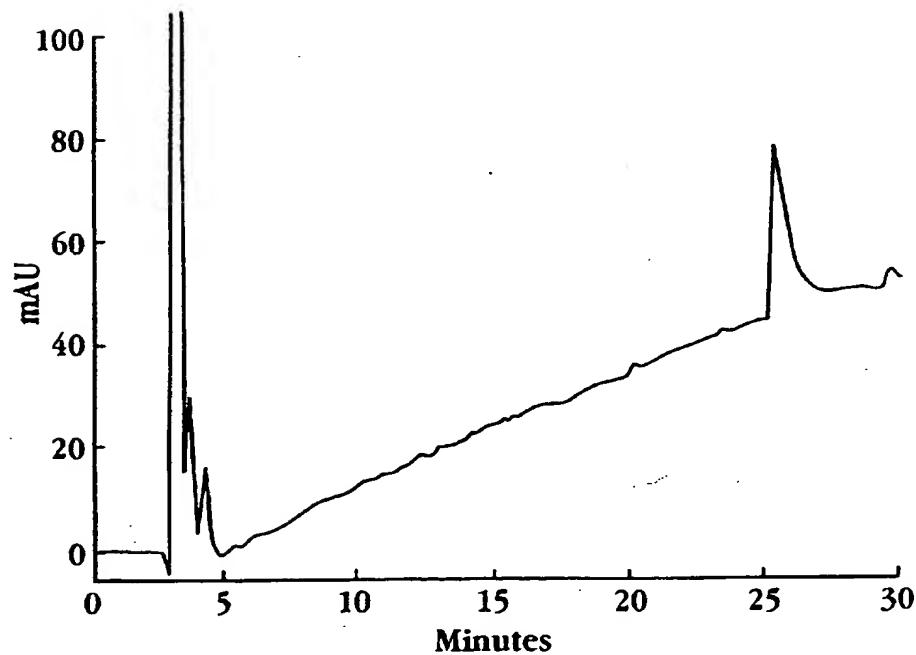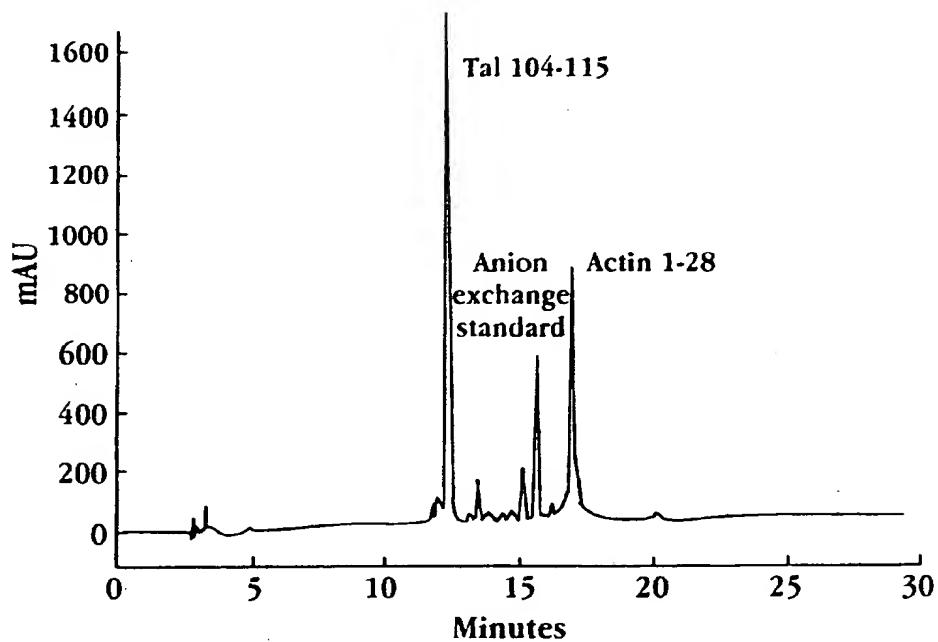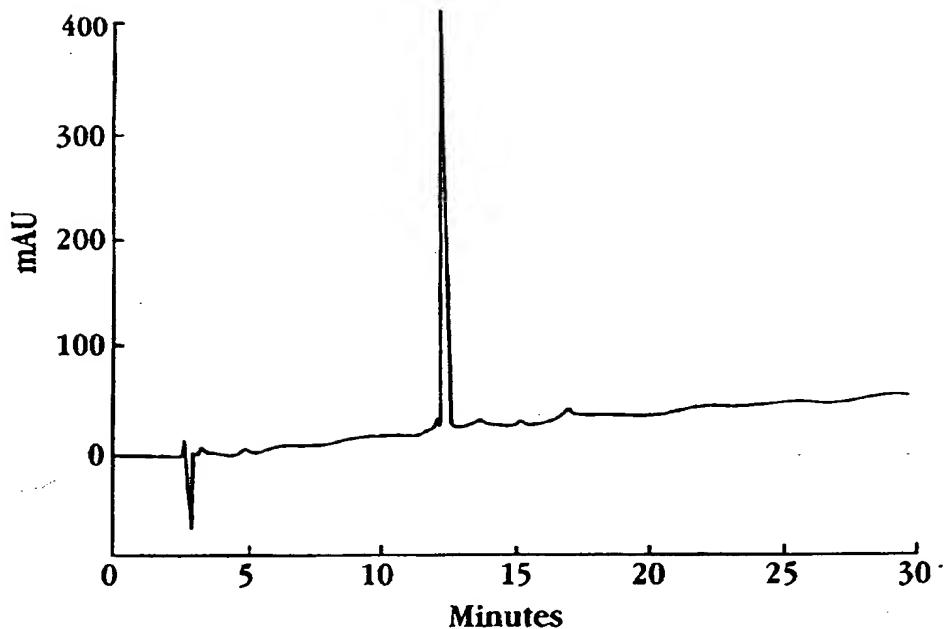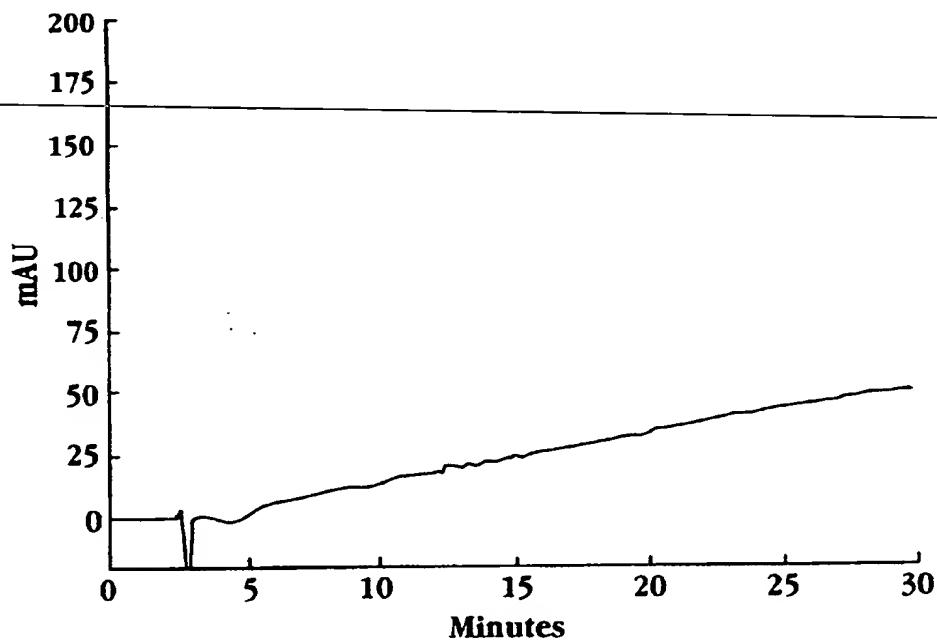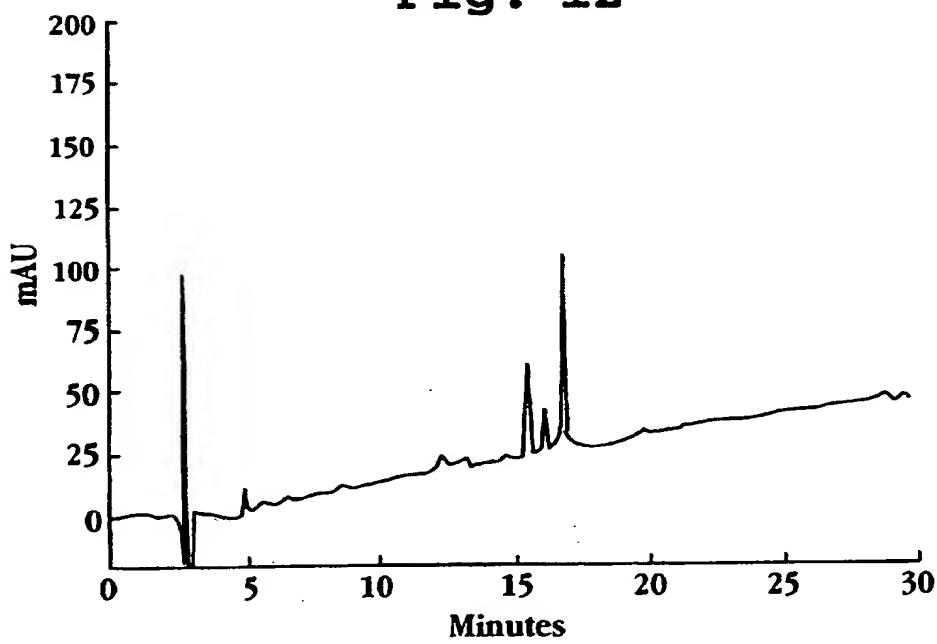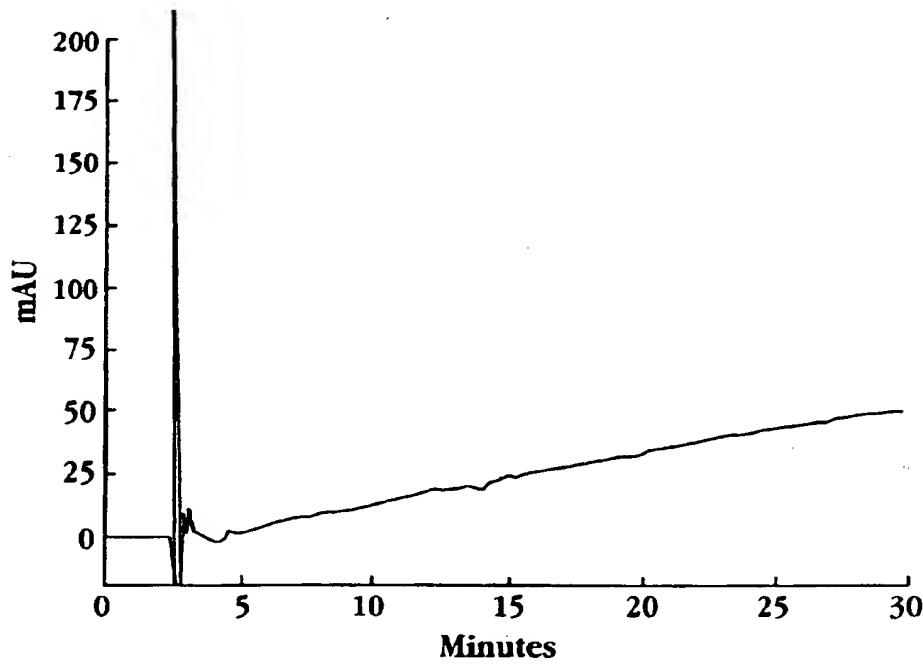
Fig. 18A

Fig. 18B

Fig. 18C

Fig. 18D

Fig. 18E

Fig. 18F

Fig. 18G
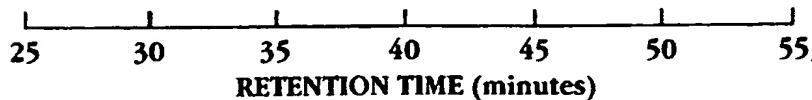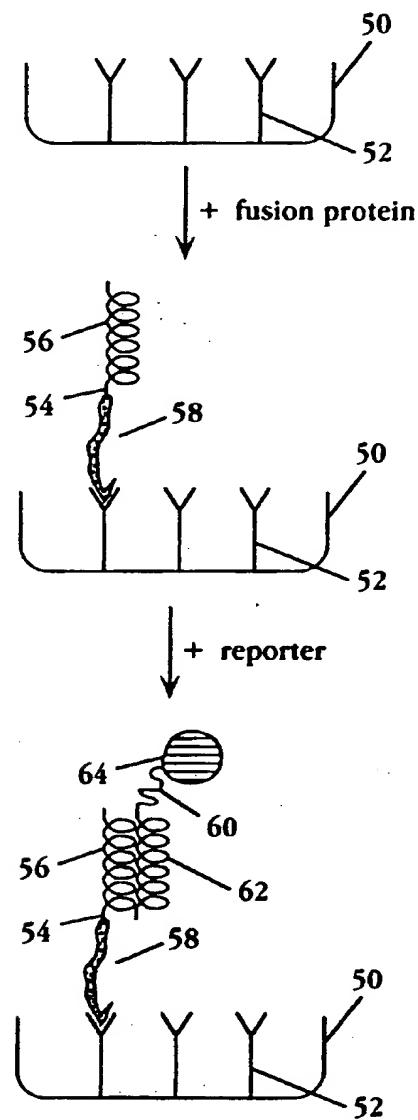
ABSORBANCE (210nm)

25   30   35   40   45   50   55
RETENTION TIME (minutes)

Fig. 19

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 21/06, 21/04; C07H 21/02, 21/04; C07K 16/00

US CL :435/69.1, 69.7, 71.1; 536/23.1, 23.4, 24.1; 530/ 388.23, 388.24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 71.1; 536/23.1, 23.4, 24.1; 530/ 388.23, 388.24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WOLBER. V. et al. A Universal Expression-Purification System Based on the Coiled-Coil Interaction of Myosin Heavy Chain Bio/Technology August 1992. Volume 10. pages 900-904, see entire article.	1-25
P, A	WINDER. S. J. et al. Protein Production in Three Different Expression Vectors from a Single Polymerase Chain Reaction Product. Analytical Biochemistry. 23 October 1995. Volume 231. Number 1. pages 271-273, see entire article.	1-25
A	TABISH. M. et al. Exclusive Expression of C. elegans osm-3 Kinesin Gene in Chemosensory Neurons Open to the External Environment. Journal of Mol. Biol. 31 March 1995. Volume 247. Number 3. pages 377-389, especially pages 382-383.	1-25

| [X] | Further documents are listed in the continuation of Box C. | | [ ] | See patent family annex. |

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
| 11 DECEMBER 1996 | 13 JAN 1997 |

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Form PCT/ISA/210 (second sheet)(July 1992)*